REMARKS

Claims 36-38, and 42-49 are pending in the application. Claims 36, 37 and 42-49 stand rejected as discussed below. New claims 50-52 have been added by amendment as shown above.

The specification has been amended as indicated above to explicitly describe compositions comprising antibodies to RANKL. The application as filed teaches that antibodies to RANKL can be used for "interfering with RANKL signaling (antagonistic or blocking antibodies), as components of diagnostic or research assays for RANKL or RANKL activity, or in affinity purification of RANKL" (see the specification at page 27, lines 31-34). It is self-evident that for such purposes the antibodies would be present in a composition. Accordingly, the application as filed inherently discloses compositions containing antibodies to RANKL. As explained in *In re Smythe* (178 U.S.P.Q. 279, 285 (C.C.P.A. 1973); copy enclosed as Exhibit A), an amendment to a patent application is not new matter if the subject matter of the amendment was inherently disclosed in the application as filed. This amendment to the specification therefore does not constitute the addition of new matter to the application.

The preambles of claims 36 and 42 have been amended as advised by the examiner to recite "A purified antibody" rather than "An antibody." Support for this change is found in the specification. For example, methods for the purification of antibodies is described at page 28, lines 23-28. Claim 36 has been amended further to recite an "antibody that specifically binds" rather than an "antibody that binds" human RANKL. This amendment is supported as discussed below in the section dealing with the rejections under 35 U.S.C. § 112, second paragraph. The amendments to claims 36 and 42 do not constitute the addition of new matter to the application.

The examiner has indicated that claim 38 would be allowable if amended so that it no longer depends from a rejected base claim. In view of the above amendments and the comments below, applicant believes that claim 38 is allowable in its present form. Nonetheless, in order to expedite the prosecution of this application, claim 38 has been amended as suggested to convert it into an independent claim. The amendments to claim 38 do not add any new matter to the application.

Support is found throughout the specification for new claims 50 and 51, for example, at page 12, lines 5-7; page 18, lines 2-3; and page 27, line 26 to page 28, line 29. Support for new claim 52 is found in the specification as described for new

claims 50 and 51, and further at page 12, first paragraph, as amended above. New claims 50-52 do not constitute the addition of new matter to the application.

Objection to the Specification

The examiner has objected to the specification on the ground that there is no antecedent basis for a composition comprising an antibody to RANKL of SEQ ID NO:13. To rectify this, she has requested that appropriate correction be made to the specification. Accordingly, the specification has been corrected as shown above, thus the examiner is respectfully requested to remove this ground for objection to the specification.

Rejections under 35 U.S.C. § 101

Claims 36 and 42 have been rejected over the examiner's position that the claims do not differentiate the claimed subject matter from antibodies as they exist naturally. She has suggested that this be remedied by amending the preambles of these two claims to recite "isolated" or "purified" antibodies. Claims 36 and 42 thus have been amended as shown above to refer to "purified antibodies." Accordingly, this ground for rejection is now moot and its removal is respectfully requested.

Rejections under 35 U.S.C. § 112, Second Paragraph

Claims 36, 37 and 42-49 stand rejected under 35 U.S.C. § 112, second paragraph. The remainder of the claims rejected under this provision depend directly or indirectly from claims 36 or 42, thus the discussion below applies to these remaining claims by virtue of their dependency.

The examiner has rejected the claims under 35 U.S.C. § 112, second paragraph because the specification does not define "specifically binds," and because she considers it unclear what the difference in scope is between "binds" and "specifically binds." Prior to the above amendments, the preamble of claim 36 recited "An antibody that binds..." while that of claim 42 recited "An antibody that specificially binds...." Claim 36 as amended above now refers also to antibodies that "specifically bind," thus eliminating the unintended implication that claims 36 and 42 cover antibodies that differ in scope as regards their specificity. In view of this amendment to claim 36, the second of these two concerns is rendered moot.

In the present instance, the specification taken as a whole, together with the knowledge of those skilled in the art, makes it readily apparent what is meant by the term "specifically binds" as it is used in the present claims.

The application does not set forth an explicit definition of "specifically binds," but the term nonetheless would be understood by those skilled in the art. In considering this issue, it should be kept in mind that there is no requirement that every word or phrase recited in a claim be extracted verbatim from the body of the specification. As noted in M.P.E.P. § 2163.02, "[t]he subject matter of the claim need not be described literally (i.e., using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement." The MPEP explains also that the courts have held that "[a] patent need not teach, and prefereably omits, what is well known in the art" (M.P.E.P. § 2164.01).

As of the filing date of this application, the concept of antibody specificity was understood as follows: "[t]he specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen, or immunogen) and any other antigen one might test" (Paul W.E., *Fundamental Immunology*, 2nd Edition, Raven Press, NY, 1989; excerpt attached as Exhibit B). This concept is basic in the field of immunology, and one skilled in the art would readily understand that if an antibody "specifically binds" a given antigen, this means that it binds with higher affinity to that antigen than to another antigen against which it is being tested. Measuring the binding affinity of an antibody is, of course, a matter of routine for those skilled in the art.

The specification discloses the amino acid sequences for two species of RANKL polypeptides, namely human and mouse RANKL. Given this context, it would be self-evident to one skilled in the art that the term "specifically binds," as used herein means that the claimed antibodies will bind with higher affinity to human RANKL than to mouse RANKL. By using the human and mouse RANKL proteins provided herein as reagents, an immunologist of ordinary skill would have been able to apply standard techniques to prepare antibodies specific for human RANKL as of the filing date of this application. After all, it was well-known at that time that antibodies are capable of distinguishing related proteins that differ by as little as one amino acid (see, for example, Lederman et al., *Mol Immunol* 28(11):1171-1181 (1991), copy attached as Exhibit C); and Abaza and Atassi, *J Prot Chem* 11(5):433-444 (1992), copy attached as Exhibit D). Accordingly, the meaning of "specifically binds" as used in claims 36 and 42 is clear. In view of these considerations, the examiner is asked to withdraw the rejections of claims 36, 37 and 42-49 under 35 U.S.C. § 112, second paragraph.

It should be noted further that in light of the above discussion, the amendment of claim 36 to recite "specifically binds" does not constitute the addition of new matter to the application.

Rejections under 35 U.S.C. § 102(e)

Claims 36, 37 and 42-49 stand rejected as being anticipated by Gorman et al. (U.S. Patent No. 6,525,180). Gorman et al. discloses mouse RANKL and contains claims to antibodies that specifically bind mouse RANKL. The examiner has assumed that the antibodies taught by Gorman et al. would cross-react with human RANKL because human and murine RANKL share 84% amino acid homology. However, as noted above, this degree of similarity by no means ensures cross-reactivity. Yet based on the assumption that the antibodies would cross-react, the examiner has concluded that the claimed antibodies and those taught by Gorman et al. are the same. However, the antibodies of Gorman et al. and the antibodies covered by the claims as amended above are not the same.

Gorman et al. does not describe a species of antibody that specifically binds human RANKL polypeptides having the amino acid sequences disclosed herein. Since Gorman et al. fails to disclose human RANKL sequences, it does not teach how to make antibodies that meet the limitations of the present claims, which cover only antibodies that specifically bind human RANKL. Gorman et al. discloses mouse RANKL for use in raising antibodies, but one would not expect such antibodies to bind *specifically* to human RANKL. Moreover, one relying on Gorman et al. would not be able to identify antibodies that specifically bind human RANKL because this reference does not disclose human RANKL sequences. Accordingly, Gorman et al. does not anticipate the antibodies described in the claims 36, 37 and 42-49, and the examiner therefore is asked to remove the rejection under 35 U.S.C. § 102(e) over this reference.

The applicant notes further that the claimed antibodies are not obvious under 35 U.S.C. § 103 over the disclosure of Gorman et al. Gorman et al. does not disclose or suggest the amino acid sequence of human RANKL, thus does not provide any means for preparing or identifying the antibodies claimed herein. Accordingly, the antibodies of claims 36, 37 and 42-49 are not obvious over this reference.

CONCLUSION

Claims 36-38 and 42-52 now are pending in the application. In view of the above remarks and amendments to the specification and claims, these claims are believed to be in condition for allowance. Notification to this effect is respectfully requested. If the examiner has any further concerns regarding this application, she is urged to contact the undersigned at her direct dial phone number given below.

Respectfully submitted,

Diana K. Sheiness

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Reg. No. 35,356
(206) 265-4818
Date: July 2, 2003

Correspondence address:

22932

dertaking in the sum of $5,000.00 to compensate defendant for such costs and damages as it may incur if it is held ultimately that defendant was wrongfully restrained or enjoined, it is

Ordered, that pending the trial and final determination of this case, defendant, its agents, officers, servants, employees, attorneys and all persons in active concert with any of them, be and hereby are enjoined and restrained from manufacturing, selling, offering to sell, advertising or distributing, any knit caps or other items of merchandise which bear or display thereon or on bags, tags, packages or displays associated therewith, any reproduction, counterfeit, copy or colorable imitation of either (1) the word "BRUINS" or (2) the "B" device shown by U. S. Trademark Registration No. 872,363 granted July 1, 1969.

Court of Customs and Patent Appeals

In re SMYTHE AND SHAMOS

No. 8855 Decided June 28, 1973

PATENTS

1. Court of Customs and Patent Appeals — Briefs (§28.05)

Court of Customs and Patent Appeals — Record (§28.30)

First patent's disclosure, incorporated into second patent by reference, is not to be ignored merely because first patent was not included in court record, there being no reason to doubt accuracy of quotation from first patent as contained in applicants' brief.

2. Claims — Broad or narrow — In general (§20.201)

Construction of specification and claims — Comparison with other claims (§22.40)

skilled in the art to compounds which it is later desired to claim; mere omission of claim limitations does not suggest omission of steps or parts.

3. Specification — Sufficiency of disclosure (§62.7)

Court cannot agree that in every case where description of invention in specification is narrower than that in claim there has been a failure to fulfill description requirement in 35 U.S.C. 112; each case must be decided on its own facts; thus, instant question is whether application originally filed clearly conveyed in any way to those skilled in the art, to whom it is addressed, the information that applicants invented analysis system with an inert fluid as segmentizing medium; if it did, applicants made a written description of invention within meaning of first paragraph of section 112.

4. Specification — Sufficiency of disclosure (§62.7)

Case is not one where there is unpredictability such that applicants' description of air or other inert gas would not convey to one skilled in the art knowledge that applicants invented an analysis system with a fluid segmentizing medium; in other cases, particularly, but not necessarily, chemical cases, where there is unpredictability in performance of certain species or subcombinations other than those specifically enumerated, one skilled in the art may be found not to have been placed in possession of a genus or combination claimed at a later date in prosecution of patent application; it is predictability of elements, be they chemical or mechanical, which is determinative; since broader concept of using "inert fluid" would naturally occur to one skilled in the art from reading applicants' description of use and functions of segmentizing media specifically described, there is no basis for denying applicants claims which recite segmentizing medium broadly as "inert fluid."

5. Amendments to patent application — New matter (§13.5)

Interference — Interference in fact (§41.40)

Interference — Reduction to practice — Constructive reduction (§41.755)

Specification — Sufficiency of disclosure (§62.7)

By disclosing in patent application a device that inherently performs a function, operates according to a theory, or has an advantage, applicant necessarily discloses that function, theory, or advantage even though he says nothing concerning it; application may later be amended to recite function, theory, or advantage without introducing new matter; rule applies in context of ex parte rejection under description requirement of first paragraph of 35 U.S.C. 112, the right to make a count in an interference, the

right to rely upon prior application under section 120 which complies with requirements of first paragraph of section 112, or a new matter rejection of claims.

6. Claims — Indefinite — In general (§20.551)

Claims are not rejected under first paragraph of 35 U.S.C. 112 because they recite "fluid" which includes some "liquids" which might not work since such liquids would be predictably inoperative and thus would never be selected by one skilled in the art; to require applicants to exclude such inoperative materials would cause failure to comply with second paragraph of section 112 because claims would be so detailed as to obscure the invention.

Particular patents—Analysis Apparatus

Smythe and Shamos, Automatic Analysis Apparatus and Method, claims 34, 37 to 40, 42 to 44, and 47 to 50 of application allowed.

Appeal from Board of Appeals of the Patent Office.

Application for patent of William J. Smythe and Morris H. Shamos, Serial No. 369,695, filed May 25, 1964; Patent Office Group 170. From decision rejecting claims 34, 37 to 40, 42 to 44, and 47 to 50, applicants appeal. Reversed; Baldwin, Judge, dissenting in part with opinion.

ERIC P. SCHELLIN and SCHELLIN & HOFFMAN, both of Arlington, Va., for appellants.

S. WM. COCHRAN (RAYMOND E. MARTIN of counsel) for Commissioner of Patents.

Before MARKEY, Chief Judge, RICH, BALDWIN, and LANE, Associate Judges, and WATSON, Judge, United States Customs Court, sitting by designation.

RICH, Judge.

This appeal is from the decision of the Patent Office Board of Appeals, adhered to on reconsideration, affirming the rejection of claims 34, 37-40, 42-44, and 47-50 of appellants' application serial No. 369,695, filed May 25, 1964, entitled "Automatic Analysis Apparatus and Method." We reverse.

The Invention

The invention relates to a continuous, automatic analysis system wherein discrete liquid samples, perhaps containing blood or other body fluids, are successively introduced into an apparatus as a continuous stream, the individual samples being separated by a segmentizing medium which, as originally claimed and as taught by the specification, is "air or other gas which is inert to the liquid" sample transmitted. The appealed claims are directed to both method and apparatus.

In the analysis apparatus a chemical reagent is automatically added to each discrete liquid sample to produce a color reaction indicative of the particular constituent in the sample to be tested, and the samples with the intervening portions of segmentizing medium are passed through the sight passageway of a flow cell as a continuous stream. The sight passageway forms part of a colorimetric analysis apparatus. Leading segments of the liquid samples, which are arranged in duplicate one following another, perform, along with the segmentizing medium, a cleansing function and each following segment has a volume at least equal to that of the sight passageway. When the sight passageway of the flow cell is fully occupied by the liquid sample to be analyzed, a recorder for the analysis, which receives its input from the colorimeter, is made operational.

Representative claims, for the purpose of dealing with the rejections, are as follows (emphasis ours):

> 34. A method of automatic quantitative analysis of a plurality of liquid samples each disposed in a respective container, wherein said samples are off-taken by an off-take device and are transmitted successively as a flowing stream to an analytical device including a flow cell having a sight passageway, said method including:
>
> for each sample container in succession, coupling said off-take device to such sample container, and in alternation therewith, to a source of an *inert fluid immiscible with said liquid samples*, thereby to off-take a segment of each of said liquid samples and intermediate segments of the inert fluid;
>
> transmitting said segments of the liquid samples and inert fluid as a flowing stream to said analytical device; and
>
> passing said flowing stream including segments of both the liquid samples and inert fluid through the sight passageway of the flow cell, the volume of at least one homogeneous portion of each liquid sample being at least equal to the volume of the sight passageway of the flow cell.
>
> 47. A method of automatic quantitative analysis of a plurality of liquid samples each disposed in a respective container, wherein said samples are off-taken by an off-take device and are transmitted successively as a flowing stream to an analytical device including a colorimeter having a flow cell with a sight passageway, said method including;
>
> for each sample container in succession, coupling said off-take device to such sample

right to rely upon prior application under section 120 which complies with requirements of first paragraph of section 112, or a new matter rejection of claims.

6. Claims — Indefinite — In general (§20.551)

Claims are not rejected under first paragraph of 35 U.S.C. 112 because they recite "fluid" which includes some "liquids" which might not work since such liquids would be predictably inoperative and thus would never be selected by one skilled in the art; to require applicants to exclude such inoperative materials would cause failure to comply with second paragraph of section 112 because claims would be so detailed as to obscure the invention.

Particular patents—Analysis Apparatus

Smythe and Shamos, Automatic Analysis Apparatus and Method, claims 34, 37 to 40, 42 to 44, and 47 to 50 of application allowed.

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S. WM. COCHRAN (RAYMOND E. MARTIN of counsel) for Commissioner of Patents.

Before MARKEY, Chief Judge, RICH, BALDWIN, and LANE, Associate Judges, and WATSON, Judge, United States Customs Court, sitting by designation.

RICH, Judge.

This appeal is from the decision of the Patent Office Board of Appeals, adhered to on reconsideration, affirming the rejection of claims 34, 37-40, 42-44, and 47-50 of appellants' application serial No. 369,695, filed May 25, 1964, entitled "Automatic Analysis Apparatus and Method." We reverse.

The Invention

The invention relates to a continuous, automatic analysis system wherein discrete liquid samples, perhaps containing blood or other body fluids, are successively introduced into an apparatus as a continuous stream, the individual samples being separated by a segmentizing medium which, as originally claimed and as taught by the specification, is "air or other gas which is inert to the liquid" sample transmitted. The appealed claims are directed to both method and apparatus.

In the analysis apparatus a chemical reagent is automatically added to each discrete liquid sample to produce a color reaction indicative of the particular constituent in the sample to be tested, and the samples with the intervening portions of segmentizing medium are passed through the sight passageway of a flow cell as a continuous stream. The sight passageway forms part of a colorimetric analysis apparatus. Leading segments of the liquid samples, which are arranged in duplicate one following another, perform, along with the segmentizing medium, a cleansing function and each following segment has a volume at least equal to that of the sight passageway. When the sight passageway of the flow cell is fully occupied by the liquid sample to be analyzed, a recorder for the analysis, which receives its input from the colorimeter, is made operational.

Representative claims, for the purpose of dealing with the rejections, are as follows (emphasis ours):

34. A method of automatic quantitative analysis of a plurality of liquid samples each disposed in a respective container, wherein said samples are off-taken by an off-take device and are transmitted successively as a flowing stream to an analytical device including a flow cell having a sight passageway, said method including:

for each sample container in succession, coupling said off-take device to such sample container, and in alternation therewith, to a source of an *inert fluid immiscible with said liquid samples*, thereby to off-take a segment of each of said liquid samples and intermediate segments of the inert fluid;

transmitting said segments of the liquid samples and inert fluid as a flowing stream to said analytical device; and

passing said flowing stream including segments of both the liquid samples and inert fluid through the sight passageway of the flow cell, the volume of at least one homogeneous portion of each liquid sample being at least equal to the volume of the sight passageway of the flow cell.

47. A method of automatic quantitative analysis of a plurality of liquid samples each disposed in a respective container, wherein said samples are off-taken by an off-take device and are transmitted successively as a flowing stream to an analytical device including a colorimeter having a flow cell with a sight passageway, said method including;

for each sample container in succession, coupling said off-take device to such sample

container, and in alternation therewith, to a source of an *inert gas* immiscible with said liquid samples, thereby to off-take a segment of each of said liquid samples and intermediate segments of the inert gas;

transmitting said segments of the liquid samples and inert gas as a flowing stream to said analytical device;

passing said flowing stream including segments of both the liquid samples and inert gas through the sight passageway of the flow cell, the volume of at least one homogeneous portion of each liquid sample being at least equal to the volume of the sight passageway of the flow cell;

measuring the optical density of the liquid samples passing through the sight passageway of the flow cell; and

interrupting the operation of said recorder except when said portion of each sample having a volume at least equal to the volume of the sight passageway of the flow cell is in the sight passageway.

Summary of Prior Art and Rejections

The following three patents were relied on as prior art:

Skeggs 2,797,149 June 25, 1957
Skeggs 2,879,141 Mar. 24, 1959
Baruch 3,193,358 July 6, 1965 (filed July 2, 1962)

Claims 34, 37-40, 42, 43, and 48-50 have been rejected for obviousness under 35 U.S.C. 103 on Skeggs '141 in view of Skeggs '149 and further in view of Baruch. As will be discussed in greater detail shortly, the examiner and the board rely particularly upon the language of claims 9 and 10 of Skeggs '149 for a teaching of what appears to be a critical limitation of these claims.

Claims 34, 37-40, 43, and 44 were rejected under 35 U.S.C. 112, paragraph one, for alleged failure to describe the invention insofar as the term "inert fluid" encompasses liquids, since the specification and original claims refer only to "air or other gas which is inert to the liquids transmitted" as the analysis samples.

Claims 47-50 were rejected under 35 U.S.C. 112, paragraph one, it being alleged that the specification does not enable one skilled in the art to use an "inert gas" as a segmentizing medium in the invention.

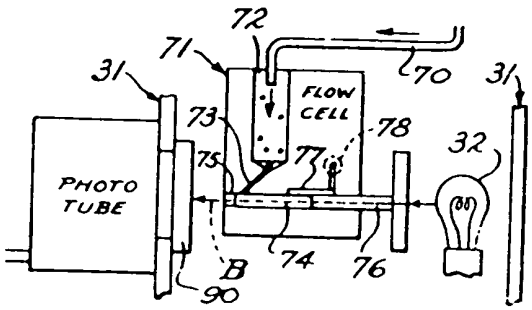
Opinion

The rejections and the positions of the parties will now be dealt with.

The Section 103 Rejection

This is the type of case where the invention resides in the discovery that an element or step which allegedly has always been included in prior art apparatus or method can be omitted, not merely with omission of its function but with improved results. Appellants admit, and the prior art of record establishes, that the *general* apparatus for performing appellants' method invention is known. Appellants contend, however, that the prior art apparatus and method always provided for what is called "venting" of the segmentizing medium, or "debubbling," just prior to passing the successive liquid samples through the sight passageway in the flow cell.

Although there is some dispute about the teachings of the Skeggs '149 patent, the general nature of the invention and the prior art practice of venting or debubbling is illustrated by reference to a portion of Fig. 3 of that patent:



Skeggs '149 discloses automatic apparatus adaptable to blood analysis having sample-feeding apparatus similar to appellants'.[1] Liquid samples flowing through a tube in a continuous stream, the samples being separated in the tube by air as a segmentizing medium, are supplied to a colorimeter, which, with a recorder, translates color changes into a record of the amount of a given ingredient in each sample. On the way to the colorimeter a reagent is mixed with each sample to produce a color reaction which can be measured by the colorimeter. The reagent-treated samples with the segmentizing medium, air, interspersed therebetween, together flow as a segmented liquid stream "into a fluid line 70 leading to a transparent plastic flow cell 71 provided with an *open chamber.*" (Emphasis ours.) "A communicating duct 73 leads from the lower end of *open chamber 72* to a horizontal cylindrical passage 74" (emphasis ours) wherein the photometric analysis is performed by recording the variations in light received by the phototube from the light source 32. Venting of the segmentizing air medium from the stream is claimed in claim 10 of Skeggs '149. The open chamber 72 in the above figure is the point at which this venting takes place. Presumably the dots shown in the open chamber are the draftsman's way of representing rising

[1] Skeggs '149 issued to Technicon International, Ltd., a New York Corporation, and Skeggs '141 issued to Technicon Instruments Corporation, a New York corporation, which appears to be prosecuting the present application.

bubbles of the segmentizing medium, which is a gas entrained in a liquid.

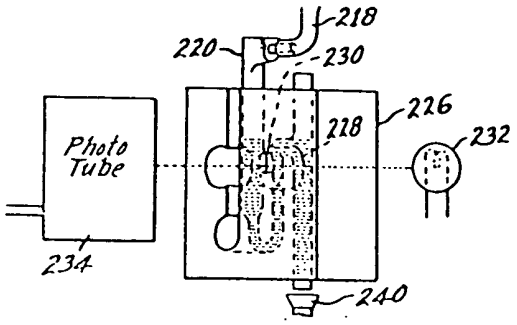
Relating this to the invention here, appellants' discovery is that it is desirable to *omit* the prior art step of venting the air or other gas, i.e., "debubbling" the segmented stream of fluid samples. Appellants argue that they were the first to discover the advantages of omitting the debubbling step. They contend that the prior art cited by the Patent Office nowhere suggests that venting or debubbling can be dispensed with.

Appellants' brief explains the advantages of not debubbling as follows:

> 2. Prior to the invention of Appellants, it was generally the practice to vent or "debubble" the segmentizing medium just prior to passing the successive liquid sample[s] through a sight passageway included in the flow cell. * * * The practice of such venting or "debubbling" resulted in the leading and trailing portions of adjacent liquid samples becoming contiguous. As a result, intermixing occurred between such contiguous portions or successive liquid samples while passing from the "debubbling" apparatus. The passage of such intermixed portions resulted in contamination of the sight passageway of the flow cell. To alleviate the problem an uncontaminated portion of a successive adjacent liquid sample, therefore, had to be passed to "wash" the sight passageway before meaningful analysis could be effected on the remaining uncontaminated portion of such successive samples. As a result, the processing rate of the liquid samples was limited and, also, the initial volume of a liquid sample required for analysis was excessive. These limitations of the prior art apparatuses were due directly to the intermixing of successive liquid samples when moving between the "debubbler" portion of the apparatus and through the sight passageway of the flow cell.
>
> 3. The present invention overcomes these prior art limitations and provides for a substantially increased processing rate and, also, a significant reduction in the initial volume of samples required for analysis. These results are achieved by maintaining the integrity of the individual liquid samples in the continuous stream while passing through the sight passageway of the flow cell.

With this as background, we continue with a description of the other Skeggs reference, '141.

The question with respect to Skeggs '141 is whether or not it discloses venting. The Patent Office view is that no venting is shown. We disagree. The following portion of Fig. 8 shows the flow cell and related parts. It is described as a "diagrammatic representation":



We agree with the solicitor that the stream passing through line 218 is an air-segmented liquid stream. The question is whether the flow cell 220 is so constructed as to vent the air from the stream before the sample is analyzed.

Skeggs '141 contains, in addition to what the above drawing shows, the following statement (emphasis ours):

> The colored mixture flows into the flow cell 220, *which preferably is of the type illustrated and claimed in the copending application of Andres Ferrari, Jr.*, Serial No. 516,300, filed June 17, 1955, and assigned to the assignee hereof. The flow cell 220 is mounted in a holder 226 provided with a light passageway 228 having a constricted opening 230 adjacent one arm of the flow cell 220. Light passing from the light source 232 through the restricted light aperture 230 is transmitted through the colored mixture in the flow cell 220 to a photoelectric device or photo tube 234.

Does this disclose a vented flow cell, though no venting is mentioned in the above quotation? We think it does. Appellants have not conclusively wrapped up their proofs as they might have because, as correctly noted by the solicitor, they did not include the Ferrari patent in the record in this court, but their brief points out that the Ferrari application is now patent No. 2,983,184, issued May 9, 1961, and they quote from the disclosure of this patent, which is incorporated by reference into Skeggs '141, as follows (omissions and insertions ours for clarity):

> * * * as the dialyzate is discharged * * * into [the] flow cell * * *, the air present between adjacent segments of said dialyzate freely escapes into the atmosphere since [the] inlet opening * * * of said cell is open thereto. Thus it will be apparent that no air can be trapped into the liquid column * * * which is subjected to the light beam.

[1] We do not consider that this disclosure, incorporated into Skeggs '141, is to be ignored merely because the Ferrari patent has not been included in the record in this court. There is

no reason to doubt the accuracy of appellants' quotation from Ferrari. We note particularly two factors indicating its reliability: it is consistent with Skeggs '141 Fig. 8 and it has not been challenged by the solicitor who had ready access to the Ferrari patent. Furthermore, the same allegations of venting in the flow cell of Skeggs '141 were made before the board, as follows:

> The flow cell 220 is of the type more particularly described in the A. Ferrari, Jr. patent No. 2,983,184, filed on July 15, 1955 * * *. The flow cell 220 includes an open ended, or vented, mixing chamber wherein the continuous stream of liquid samples directed along conduits 216 and 218 is discharged, so as to purposefully vent the air bubbles. The liquid samples, now contiguous, intermix * * *

We simply have to disagree with the board's conclusions that "Skeggs '141 does not mention the venting present in Skeggs '149" and that "The drawing in Skeggs '141 does not appear to require such venting." These statements are inconsistent with the whole disclosure of Skeggs '141, which includes the description of the structure of the Ferrari flow cell, "diagrammatically" shown in Fig. 8, incorporated by reference.

Obviousness of an unvented system as claimed by appellants therefore cannot be predicated on the disclosures of the specifications of Skeggs '149 or '141. The board admitted that venting is "present in Skeggs '149." The board and the solicitor are clearly wrong in suggesting that the flow cell of Skeggs '141 is unvented. One other basis for finding obviousness is argued, predicated on a difference which exists between claims 9 and 10 of Skeggs '149. Claim 10 reads (emphasis ours):

> 10. Apparatus for analyzing liquid samples containing a crystalloid constituent and a noncrystalloid constituent, *comprising* a dialyzer having a diaphragm and an inlet and an outlet at one side of the diaphragm for the passage of a stream of sample-containing liquid through the dialyzer at said side of the diaphragm, said dialyzer also having an inlet and an outlet at the other side of said diaphragm for the passage of streams of liquid into and out of the dialyzer at said other side of the diaphragm to form a stream of liquid containing the crystalloid substance which is diffused through the diaphragm from said first mentioned side thereof means for treating the liquid of said last mentioned stream for colorimetric analysis thereof, a colorimeter flow-cell to receive the treated liquid, tubes leading to and from the dialyzer for conducting the liquids to and from the dialyzer and to the flow-cell and means for introducing air into the tubes leading to the dialyzer for forming segmented streams of fluid composed of alternate segments of liquid and air passing through said tubes and the dialyzer and through the connections of the latter to said flow-cell, *and a vent for the escape of the air from said stream of treated liquid received in said flow-cell whereby an uninterrupted column of liquid is provided in the cell for colorimetric analysis of said liquid.*

Claim 9 of the patent is substantially identical down to the emphasized clause, which is omitted in claim 9. Based on this difference, and a similar omission of venting in other claims such as 20 and 22, the Patent Office argues that *a comparison of claims*

> * * * would clearly suggest to the plant engineer that the flow of segmented streams "to said flow cell" encompasses flow thereof through such cell.

We cannot agree. We do not believe that, either as an abstract principle or as applied to the facts of this case, the omission of the vent in one or more claims of Skeggs '149 coupled with the inclusion of the vent in another claim itself would suggest to one skilled in the art that venting may be dispensed with. The specification of Skeggs '149 just does not support such a construction; the air-segmented stream "passes into a fluid line 70 leading to a transparent plastic flow cell 71 *provided with an open chamber 72* * * *." (Emphasis ours.)

[2] As to the major part of the argument of the solicitor, we do not find the difference between claims 9 and 10 to suggest or indicate to one of ordinary skill in the art that "the flow cell in question can at the option of the designer or 'plant engineer' be vented or not." The practice, time out of mind, has been that claims may be drafted as broadly as the prior art will allow, and that appears to be all that Skeggs has done in patent '149. The mere omission of claim limitations does not suggest omission of steps or parts. Furthermore, claim 9 still reads on apparatus containing venting means because of the word "comprising." In re Halley, 49 CCPA 793, 296 F.2d 774, 132 USPQ 16 (1961); Swain v. Crittendon, 51 CCPA 1459, 332 F.2d 820, 141 USPQ 811 (1964). Compare the discussion of reliance upon the *absence* of limitations in claims in another context in In re Cole, 51 CCPA 919, 326 F.2d 769, 140 USPQ 230 (1964).

Accordingly the rejection of claims 34, 37-40, 42, 43, and 48-50 under § 103 is reversed.

The § 112 Rejections
"Inert Fluid"

Claims 34, 37-40, 43, and 44 describe the segmentizing medium as an "inert fluid." The board affirmed the examiner's rejection of

these claims saying, "As to the * * * term [fluid], the specification provides no antecedent basis or description of such fluids and therefore does not support the claims." The board noted that "additional structure is necessary to adapt the disclosed contribution to employ fluids other than air." The board further noted that the term "fluid" is "so broad

The solicitor states that the "Board's rationale makes it clear that it regarded 35 U.S.C. 112, paragraph 1 as the proper statutory basis of its rejection," and particularly argues that appellants fail to *describe* their invention in their specification.[2] The solicitor, explaining the basis of this rejection on the facts of this case, takes the position that "where the description of the invention is narrower than the scope of protection sought by the claims * * * the claims may be rejected under Section 112, paragraph 1, even though the term 'fluid' embraces both 'liquid' and 'gas' * * * and even though it 'would not encompass undue experimentation to arrive at a satisfactory method and structure to employ liquid and gases other than air' * * *." As the solicitor notes in his brief,

> The important point here is that appellants did not recite the use of 'fluid' broadly as a segmenting medium in describing their invention. * * *
>
> Insofar as the term 'fluid' in claims 34, 37-40, 43 and 44 encompasses liquids, there is no *description* thereof in appellants' specification.

[3] We cannot agree with the broad proposition, apparent in the above quoted language, that in every case where the description of the invention in the specification is narrower than that in the claim there has been a failure to fulfill the description requirement in section 112. Each case must be decided on its own facts. The question which must be answered is whether the application originally filed in the Patent Office clearly conveyed in any way to those skilled in the art, to whom it is addressed, the information that appellants invented the analysis system with an inert fluid as the segmentizing medium. See In re Ruschig, 54 CCPA 1551, 379 F.2d 990, 154 USPQ 118 (1967). If it did, then appellants have made a written description of their invention within the meaning of the first paragraph of 35 U.S.C. 112. Let us look at the description of the invention in the original application.

The segmentizing medium serves the functions, appellants' specification tells us, of "separating one [liquid] sample from another in the apparatus and for washing the conduits and the flow cell * * *." The essential function of separating discrete samples from each other is performed because the medium takes the shape of the supply lines and the flow cell through which it passes, while to some extent resisting any force which may tend to change its volume. This quality is precisely that of a "fluid" generically and of a "liquid" in particular. The "washing" function of the segmentizing medium appears to us, indeed, to be better performed using liquid rather than gas, and the Patent Office has given us no facts which would justify a conclusion that one skilled in the art would not find the disclosure to inherently teach that it is the very characteristics of *fluids* which are needed in a segmentizing medium here. We note also that the prior art Kessler patent No. 3,047,367, issued in 1962, made of record by appellants here and before the examiner, shows the use of a *liquid* segmentizing and cleansing medium instead of "air or other inert gas" in a system entitled "Automatic Analysis With Fluid Segmentation." In that patent it is stated that:

> In accordance with the present invention and pursuant to one of the objects thereof, the use of air or other inert gas as the cleansing agent is dispensed with and replaced by a liquid, in order to obviate certain difficulties which may be encountered when air or other compressible fluids are employed as the cleansing agents.

We believe that the use of an inert *fluid* broadly in this invention would naturally occur to one skilled in the art reading the description of the use of air or other gas *as a segmentizing medium* to separate the liquid samples. While fluid is a broader term, encompassing liquids, as noted by the solicitor, the specification clearly conveys to one skilled in the art that in this invention the characteristics of a fluid are what make the segmentizing medium work in this invention.

[4] This is not a case where there is any unpredictability such that appellants' description of air or other inert gas would not convey to one skilled in the art knowledge that appellants invented an analysis system with a fluid segmentizing medium. In other cases, particularly but not necessarily, chemical cases, where there is unpredictability [3] in performance of certain species or subcombinations other than those specifically enumerated, one skilled in the art may be found not to have

[2] The board may have also treated the rejection of these claims under § 112 under the "enablement" section of the first paragraph, but the solicitor has narrowed the rejection by his argument to the "description" requirement.

[3] As we pointed out in In re Cook, 58 CCPA 1049, 1054, 439 F.2d 730, 734, 169 USPQ 298, 301 (1971), it is the predictability or the unpredictability of the elements, be they chemical or mechanical, which is determinative.

been placed in possession of a genus or combination claimed at a later date in the prosecution of a patent application. See, for example, In re DiLeone (DiLeone II), 58 CCPA 934, 436 F.2d 1033, 168 USPQ 598 (1971), and In re DiLeone (DiLeone I), 58 CCPA 925, 436 F.2d 1404, 168 USPQ 592 (1971), where Judge Baldwin, in dissent, stated at 58 CCPA at 929, 436 F.2d at 1406-07, 158 USPQ at 594-95:

> In the case before us, the Patent Office tribunals and in particular, the examiner—who must be presumed by us to be skilled in the pertinent art in the absence of evidence to the contrary—have disputed the fact that the scope of appellants' invention would be obvious from the language of the description. Keeping in mind the well-known unpredictability of the chemical sciences, I find that the examiner's objections were reasonable. Beyond asserting that they are entitled to the broad claims they are seeking, appellants have not contradicted this position. Feeling, as I do, that the description requirement should serve to assure that one of ordinary skill in the pertinent art will, in fact, be taught by a specification disclosure, I conclude that the disclosure before us does not adequately describe the subject matter being claimed. [Footnote omitted.]

Here, however, where the broader concept of using "inert fluid" would naturally occur to one skilled in the art from reading appellants' description of the use and functions of the segmentizing media specifically described, we see no basis for denying appellants the claims which recite the segmentizing medium broadly as an "inert fluid." The alternative places upon patent applicants, the Patent Office, and the public the undue burden of listing, in the case of applicants, reading and examining, in the case of the Patent Office, and printing and storing, in the case of the public, descriptions of the very many structural or functional equivalents of disclosed elements or steps which are already stored in the minds of those skilled in the arts, ready for instant recall upon reading the descriptions of specific elements or steps.

We are not saying that the disclosure of "air or other gas which is inert to the liquid" sample *by itself* is a description of the use of all "inert fluid" media. Rather, it is the description of the *properties and functions* of the "air or other gas" segmentizing medium described in appellants' specification which would suggest to a person skilled in the art that appellants' invention includes the use of "inert fluid" broadly. The Kessler patent is only some additional evidence of the knowledge of one skilled in the automatic sample analysis art, and as such it supports appellants' position that to such persons appellants' description conveys the idea of using inert fluids broadly.

A hypothetical situation may make our point clear. If the original specification of a patent application on the scales of justice disclosed only a 1-pound "*lead* weight" as a counterbalance to determine the weight of a pound of flesh, we do not believe the applicant should be prevented, by the so-called "description requirement" of the first paragraph of § 112, or the prohibition against new matter of § 132, from later claiming the counterbalance as a "metal weight" or simply as a 1-pound "weight," although both "metal weight" and "weight" would indeed be progressively broader than "lead weight," including even such an undisclosed, but obviously art-recognized equivalent, "weight" as a pound of feathers. The broader claim language would be permitted because the *description of the use and function* of the lead weight as a scale counterbalance in the *whole disclosure* would immediately convey to any person skilled in the scale art the knowledge that the applicant invented a scale with a 1-pound counterbalance weight, regardless of its composition. Likewise, we find in the facts here a description of the use and function of the segmentizing medium which would convey to one skilled in the sample-analysis art the knowledge that applicants invented a sample analyzer with an inert *fluid* segmentizing medium.

[5] Turning to the precedents, in In re Reynolds, 58 CCPA 1287, 443 F.2d 384, 170 USPQ 94, 98 (1971), this court quoted with approval the following from the opinion in Technicon Instruments Corp. v. Cole Instruments, Inc.,[4] 255 F.Supp. 630, 150 USPQ 227 (N.D. Ill. 1966), aff'd., 385 F.2d 391, 155 USPQ 369 (7th Cir. 1967):

> By disclosing in a patent application a device that inherently performs a function, operates according to a theory, or has an advantage, a patent applicant necessarily discloses that function, theory or advantage even though he says nothing concerning it. The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter.

We agree with that statement, whether, as here, in the context of an ex parte rejection under the description requirement of the first paragraph of § 112, the right to make a count in an interference, Woofter v. Carlson, 54 CCPA 917, 367 F.2d 436, 151 USPQ 407 (1966), the right to rely upon a prior application under 35 U.S.C. 120 which complies

[4] The case involved, interestingly enough, the validity of the two Skeggs '149 and '141 patents, inter alia, which were cited as § 103 references here.

with the requirements of the first paragraph of § 112, In re Lukach, 58 CCPA 1233, 442 F.2d 967, 169 USPQ 795 (1971), or a new matter rejection of the claims as discussed in Technicon.[5] See In re Welstead, 59 CCPA 1105, 463 F.2d 1110, 174 USPQ 449 (1972), a new matter case citing Fields v. Conover, 58 CCPA 1366, 1372-74, 443 F.2d 1386, 1391-92, 170 USPQ 276, 279-80 (1971), as involving the written description requirement in another context.

The board also rested the alleged failure of the specification to describe the invention on possible inclusion of inoperative embodiments of the invention using "inert fluids" which it conceived. The board stated (emphasis ours):

> The term "inert fluid" encompasses *colored* materials *adherent* to the walls of the sight tube, thus to render appellants' process inoperative, as well as liquid *wetting agents*, which appellants disclose * * * must be absent for proper operation. Thus, not only does the specification fail to support the method and apparatus claimed, where a fluid other than air is to be introduced, but the noted term is *so broad as to include inoperative fluids*. Appellants' specification, in its failure to provide antecedent basis for "inert fluid," renders it impossible for one skilled in this art to determine what classes of fluids are useful and which are not.

We can see no basis for either the board's premise that the use of the word fluid makes the claim so broad as to include inoperative fluids, or the board's conclusion that somehow any lack of antecedent basis for "inert fluid" makes it impossible to determine what classes of fluids are useful in the invention.

[6] The use here of any particular "liquids" which would be inoperative, such as the examples given by the board—"colored materials," materials "adherent to the walls of the sight tube," and "liquid wetting agents"—would be predictably inoperative in the invention and thus would never be selected by one skilled in the art. As we have said before, it is almost always possible to so construe a claim as to have it read on inoperative embodiments, In re Cook, 58 CCPA 1049, 1054, 439 F.2d 730, 734, 169 USPQ 298, 301 (1971), but the alternative of requiring an applicant to be so specific in his claims "as to exclude materials known to be inoperative and [which] even those *not* skilled in the art would not try" would result in claims which would fail to comply with 35 U.S.C. 112, second paragraph, because they would be so detailed as to obscure, rather than to particularly point out and distinctly claim, the invention. In re Myers, 56 CCPA 1129, 410 F.2d 420, 161 USPQ 668 (1969), quoted with approval in In re Anderson, 471 F.2d 1237, 176 USPQ 331 (CCPA 1973). We therefore cannot agree with the board that the rejection under the first paragraph of § 112 is any more sustainable because the broader term "fluid" includes some "liquids" which might not work.

"Inert Gas"

Claims 47-50 have been rejected under 35 U.S.C. 112, paragraph one, on the ground that insofar as the term "inert gas" used in these claims covers the use of gases other than air[6] as the segmentizing medium, the specification allegedly does not enable one skilled in the art to "make and use" the invention. The specification shows the segmentizing medium as air which is aspirated from the atmosphere surrounding the apparatus. The Patent Office position is that the use of an inert gas other than the air is not taught by the specification in a manner which would enable one skilled in the art to practice the invention.

Appellants maintain here, as they did before the Patent Office, that the Skeggs '149 patent suggests that any medium, liquid or gas, can be introduced along a pump tube by connecting the upstream side of the pump tube to the source of the medium. This Skeggs reference, as appellants point out, shows a series of openings for incoming fluid lines which lead either to a source of liquid or air, and appellants tell us that "it would not encompass undue experimentation to arrive at a satisfactory method and structure to employ liquid and gases other than air." We find ourselves in agreement with appellants. See In re Borkowski, 57 CCPA 946, 422 F.2d 904, 164 USPQ 642 (1970).

The rejections of claims 34, 37-40, 43, 44,

[5] The paragraph of the above Technicon quotation concludes with the sentence:

> In any event, the fact that the Patent Office allows such an amendment without objection thereto as new matter (within the meaning of Title 35 U.S.C., § 132) is entitled to an especially weighty presumption of correctness.

We note that here, too, the Patent Office made no new matter rejection of the language of the preliminary amendment reciting the segmentizing medium as an "inert fluid." See MPEP 608.04(b).

[6] For the purposes of considering this ground of rejection it is not necessary to decide whether the claim language "inert gas immiscible with said liquid samples" means a chemically "inert" gas or only a gas which is inert with respect to the liquid sample. At least once in the original specification the segmentizing medium is described as "air or other gas which is inert to the liquid transmitted * * *." The solicitor states that "air is not inert." For present purposes, the enablement rejection relates specifically to the use of a gas other than atmospheric air, because it cannot be merely aspirated from the surrounding atmosphere; it does not matter whether it is chemically an inert gas.

and 47-50 under 35 U.S.C. 112 are accordingly reversed.

The decision of the board is *reversed* as to all claims on appeal.

BALDWIN, Judge, dissenting in part.

I would affirm the rejection of claims 34, 37-40, 43 and 44 under the first paragraph of section 112 of the statute. The critical term in these claims, "fluid," is a generic term, which for our purposes may be considered to be made up of two major subgenera—liquids and gases. I agree that the appellants' specification adequately describes the genus of gases which meet the other claimed criteria, i.e., gases which are "inert to the sample liquid," which is how I interpret the recitation "inert gas." However, I cannot agree that the specification contains an adequate description of the genus "fluid" under any reasonable standard. In the first place, only the subgenus "inert gases" is described, and it is apparent that one skilled in this art would not infer "inert fluids" from a description of "inert gases" alone. Secondly, it cannot be questioned that any other members of the "genus" (in this case there being only one, viz. "inert liquids") is anywhere disclosed or implied in the specification. Thus description of the genus cannot be inferred from the supplied descriptions of its member species. The final possibility is a description of the genus itself, either in ipsis verbis, which clearly is not present here, or implied, such as through a complete description of the properties which define the genus, which the majority contends exists in this case. I submit that the majority is in error.

The following excerpt is sufficiently representative of the contents of the specification concerning the segmentizing medium (emphasis supplied):

> The primary object of the present invention is to improve the precision of quantitative analysis of the samples of liquid. We have discovered that this important object can be accomplished by utilizing as much as possible liquid conduits, such as Teflon tubing, which have non-wetting surfaces instead of wettable surfaces, and by washing the flow cell between the passage of successive samples therethrough with one or more *bubbles of air or other gas which is inert to the liquid transmitted* through the conduits and through the flow cell, whereby contamination of one sample by another is prevented or is negligible. Further, the sample liquid which is transmitted through the flow cell during the analysis operation, at which time a record of the analysis is made, has a volume at least as large and preferably larger than the volume of the flow cell, so that there is no *air* in the flow cell when the liquid analysis operation is being performed. More specifically, while one or more *segments or bubbles of the air or other gas* are introduced into the liquid stream for separating one sample from another in the apparatus and for washing the conduits and the flow cell, it is unnecessary to remove said *bubbles* before transmission of the treated liquid samples through the flow cell, since the segmentation of the liquid stream by *air bubbles* is such that a sufficient volume of the treated sample liquid is devoid of *air bubbles* to enable its analysis as it flows through the flow cell. By reason of the provision of a volume of non-segmented treated liquid sample sufficiently large for analysis, namely, a volume of treated liquid sample at least as large as the volume of the flow cell, blending of segments of the same liquid sample and the need for removal of a comparatively large number of *air bubbles* are obviated.

Throughout the specification, only inert gas and, more often, air, which is used in the preferred embodiment, are mentioned as segmentizing mediums. There is no further discussion of the properties which those mediums have which make them useful. Thus the two properties relied on by the majority as delineating the properties of a "fluid," i.e., (1) the ability of the medium to take "the shape of the supply lines and the flow cell through which it passes" and (2) its ability to resist "any force which may tend to change its volume," are never disclosed or discussed in the specification at all. True enough, the inert gases described by appellants inherently would possess the first property mentioned, and presumably even the most compressible of gases would qualify as having the second property "to some extent," as broadly phrased by the majority. But what the majority is saying is that the description requirement as to "inert fluids" is satisfied in this case, even though that genus is not specifically described by name, because it is impliedly described by the properties which define it, even though those properties are not specifically described by naming them, because those properties are impliedly described—by what?—by the fact that the members of the subgenus which *is* specifically described inherently possess those properties. What about those properties inherently possessed by gases which are strikingly different from those of liquids and would suggest that liquids would *not* be useful, such as density and viscosity?

More importantly, what kind of mental gymnastics are we going to force the skilled in the art to go through in order to determine what is described in a patent specification? The statute directs the specification to the skilled in the art, and that has two effects on

the requirements for its content. Of course it establishes that the intended reader is not a babe in the woods, and that therefore the specification need not be burdened with those details which are already generally known in the art. But the intended reader is *no more* than one skilled in the art. He is not some master of law or logic who can be expected to inveterately expand each nuance contained in a specification into pyramids of disclosure. It is a general level of specialized knowledge which is required of him, not genius.

As to the majority's speculation that the washing function of the segmentizing medium would be "better performed using liquid rather than gas," it is not only not supported by the record, but is also contrary to the implications of the only pertinent teachings in the record. Kessler, *whose invention it was to use a liquid segmentizing medium rather than a gas*, states as follows (emphasis supplied):

> In accordance with the present invention and pursuant to one of the objects thereof, the use of air or other inert gas as the cleansing agent is dispensed with and replaced by a liquid, in order to obviate certain difficulties which may be encountered when air or other compressible fluids are employed as the cleansing agents. In this connection, it will be understood that in most instances, *the use of air as the cleansing agent is to be preferred because it is highly effective for this purpose* and is readily available, without cost, from the ambient atmosphere. However, in certain processes, e.g., in spectral-flame analyses, the compressibility of air or other inert gas may result in pulsations of the liquid introduced into the spectral flame and thus cause the flame to flicker or be unsteady. This objectionable result is eliminated pursuant to this invention, since liquid is incompressible.

In the final analysis, I believe that support for the majority's position reduces to the existence of the Kessler patent, which, I agree, is *some* evidence that the knowledge that liquid segmentizing mediums can be used was part of the general level of skill in this art. The question becomes whether this evidence is enough on the facts of this case to establish that the use of liquid segmentizing mediums was generally known in this art. If it is, then appellants are entitled to claims directed to the use of fluids (or for that matter to liquids, which as a practical matter is the same thing here) even though appellants never mentioned those embodiments in their specification as filed, at least insofar as the description requirement is concerned. Of course, it must be *generally* known—the statute directs the specification to those skilled in the art, not just to one or two practitioners who might happen to know it.

Before us we have appellants' specification, which is very strictly limited to "air or other gas inert to the sample liquid." The segmentizing medium is nowhere described as a "medium" or a "fluid." The specification is so strictly limited as to give rise to an implication that appellants considered such a limitation important. The record before us contains twenty-five patents, twenty-four of which have disclosures which are limited to air, or other inert gas in a manner similar to appellants' disclosure. To me that indicates that the only segmentizing medium which would "naturally occur" to one skilled in the art reading appellants' specification is air, or other gas inert to the liquid samples. The mere existence of the Kessler patent, which states that the preferred segmentizing medium is gaseous in all but a few special cases, is not enough to overcome what the other evidence indicates is the general knowledge of those skilled in the art.

I would therefore affirm the rejection of claims 34, 37-40, 43 and 44.

Court of Customs and Patent Appeals

FLYNN v. EARDLEY, STOCKER, AND LONG

No. 8865 Decided June 28, 1973

PATENTS

1. Specification — Sufficiency of disclosure (§ 62.7)

It is not necessary to name claimed compounds in order to comply with first paragraph of 35 U.S.C. 112; further, preference for specific claimed compounds is not necessarily a requirement; however, specification must contain adequate direction which reasonably leads skilled in the art to compounds which it is later desired to claim; a discussion of preferences for various aspects of the invention may be one way in which to supply such direction.

Particular patents—Cephaloridine Salts

3,280,118, Eardley, Stocker, and Long, Cephaloridine Salts, awarded priority against Flynn application.

Appeal from Board of Patent Interferences of the Patent Office.

Patent interference No. 96,095 between Edwin H. Flynn, application, Serial No.

Exhibit B

FUNDAMENTAL IMMUNOLOGY

SECOND EDITION

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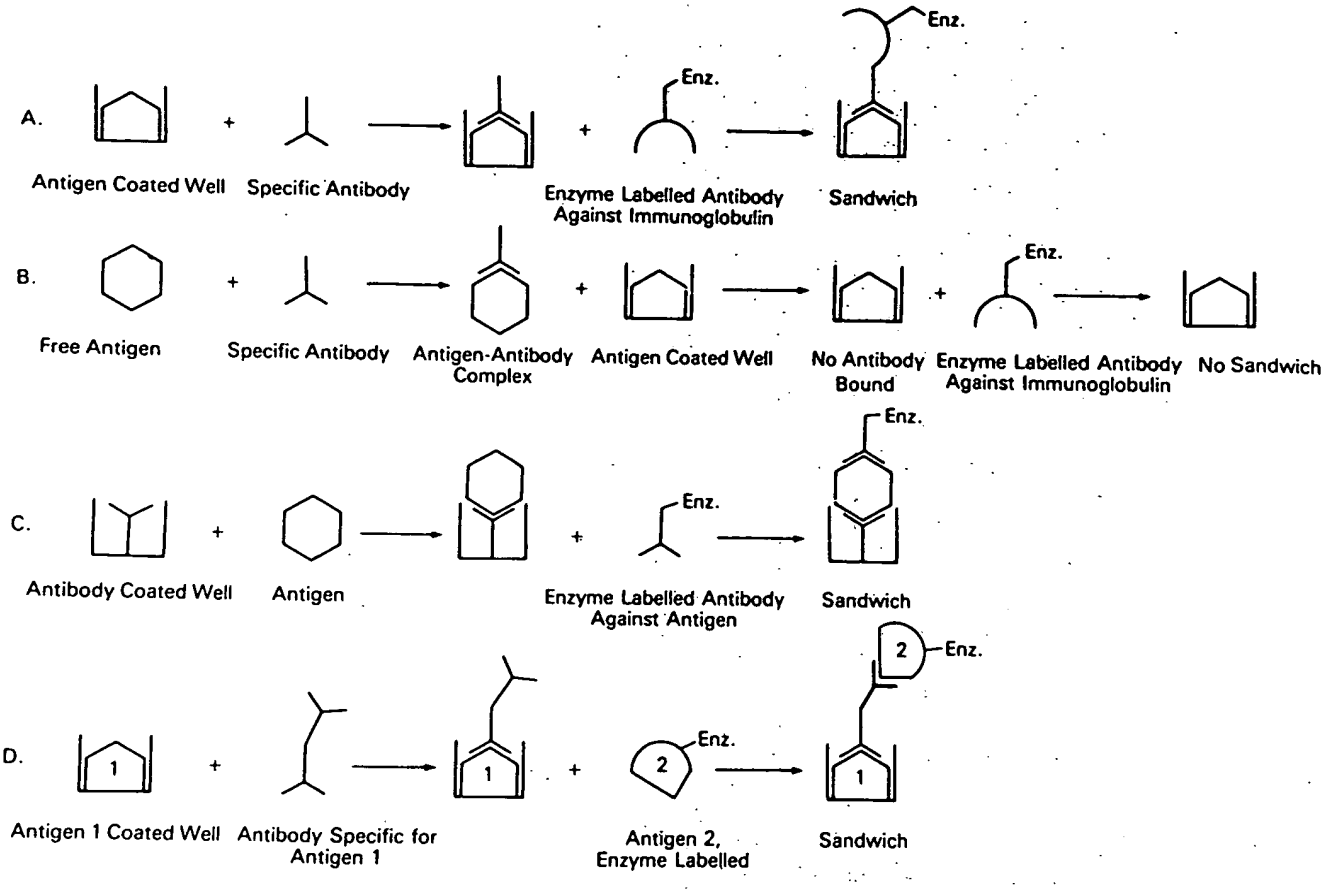

FIG. 9. Four strategies for the detection of specific antibody–antigen reactions using the ELISA technique. **A:** Direct binding. **B:** Hapten inhibition. **C:** Antigen sandwich. **D:** Antibody sandwich.

rations of idiotype, or for detection of anti-anti-idiotype (see Chapters 9 and 23). Other arrangements of antibody and antigen are also possible. Extra layers of detecting reagents can amplify sensitivity but also tend to raise the background and introduce variability.

An example of the first method described above is the detection of human antibodies to influenza virus (52) (Fig. 10). Alternate columns were coated with influenza virus or bovine albumin. Serum was added at 1/10 dilution to the top two wells of each box and serially diluted in four-fold steps from top to bottom. The last colored well indicates the titer, whereas the absence of color in the albumin-coated wells indicates the specificity. A second use of this method is for screening culture supernatants in the production of hybridoma antibodies. The sensitivity and speed of the ELISA method make it possible to screen large numbers of wells for the production of specific antibody. Clones selected by this method tend to have high antigen affinities, perhaps due to dissociation of low-affinity antibodies during the wash steps.

An important caution when using native protein antigens to coat solid-phase surfaces (Fig. 9A) is that binding to a surface can alter the conformation of the protein. For instance, using conformation specific monoclonal antibodies to myoglobin, Darst et al. (53) found that binding of myoglobin to a surface altered the apparent affinity of some antibodies more than others. This problem may be avoided by using the method of Fig. 9B.

SPECIFICITY AND CROSS-REACTIVITY

The specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen, or immunogen) and any other antigen one might test. In practice, one cannot test the whole universe of antigens, but only selected antigens. In this sense, specificity can only be defined experimentally within that set of antigens one chooses to compare. Karush (28) has defined a related term, selectivity, as the ability of an antibody to discriminate, in an all-or-none fashion, between two related ligands. Thus selectivity depends not only on the relative affinity of the antibody for the two ligands but on the experimental lower limit for detection of reactivity. For instance, an anti-carbohydrate antibody with an affinity of $10^5\ M^{-1}$ for the immunogen may appear to be highly selective, since reaction with a related carbohydrate with a 100-fold lower affinity, $10^3\ M^{-1}$, may be undetectable. On the other hand, an antibody with an affinity of $10^9\ M^{-1}$ for the homologous ligand may appear to be less selective because any reaction with a related ligand with a 100-fold lower affinity would still be quite easily detectable.

Conversely, cross-reactivity is defined as the ability to react with related ligands ther than the immunogen.

Molecular Immunology, Vol. 28, No. 11, pp. 1171–1181, 1991
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Exhibit C

0161-5890/91 $3.00 + 0.00

A SINGLE AMINO ACID SUBSTITUTION IN A COMMON AFRICAN ALLELE OF THE CD4 MOLECULE ABLATES BINDING OF THE MONOCLONAL ANTIBODY, OKT4

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Abstract—The CD4 molecule is a relatively non-polymorphic 55 kDa glycoprotein expressed on a subset of T lymphocytes. A common African allele of CD4 has been identified by non-reactivity with the monoclonal antibody, OKT4. The genetic basis for the $OKT4^-$ polymorphism of CD4 is unknown. In the present paper, the structure of the CD4 molecule from an homozygous $CD4^{OKT4-}$ individual was characterized at the molecular level. The size of the $CD4^{OKT4-}$ protein and mRNA were indistinguishable from those of the $OKT4^+$ allele. The polymerase chain reaction (PCR) was used to map the structure of $CD4^{OKT4-}$ cDNAs by amplifying overlapping DNA segments and to obtain partial nucleotide sequence after asymmetric amplification. PCR was then used to clone $CD4^{OKT4-}$ cDNAs spanning the coding region of the entire, mature CD4 protein by amplification of two overlapping segments followed by PCR recombination. The nucleotide sequence of $CD4^{OKT4-}$ cDNA clones revealed a G→A transition at bp 867 encoding an arginine→tryptophan substitution at amino acid 240 relative to $CD4^{OKT4+}$. Expression of a $CD4^{OKT4-}$ cDNA containing only this transition, confirmed that the arginine→tryptophan substitution at amino acid 240 ablates the binding of the mAb OKT4. A positively charged amino acid residue at this position is found in chimpanzee, rhesus macaque, mouse and rat CD4 suggesting that this mutation may confer unique functional properties to the $CD4^{OKT4-}$ protein.

INTRODUCTION

The CD4 molecule is a relatively non-polymorphic, 55 kDa surface glycoprotein comprised of four extracellular domains, a hydrophobic transmembrane region and a hydrophilic cytoplasmic tail (Maddon *et al.*, 1985, 1987). The NH_2-terminal extracellular domain (V1) has striking sequence homology to immunoglobulin (Ig) light chain variable regions (Maddon *et al.*, 1985). The other extracellular domains (V2–V4) are homologous to other members of the Ig gene superfamily. For example, the V3 domain is related by amino acid sequence and predicted secondary structure to the poly Ig receptor (Clark *et al.*, 1987). The CD4 molecule is expressed predominately on the cell membrane of helper T lymphocyte subsets which recognize peptide antigens bound to Class II MHC (Ia) molecules (White *et al.*, 1978; Reinherz and Schlossman, 1980; Thomas *et al.*, 1983; Dialynas *et al.*, 1983; Janeway *et al.*, 1988).

On the cell surface, CD4 expression mediates binding to Ia molecules (Doyle and Strominger, 1987) as well as functional interactions of helper and cytotoxic $CD4^+$ T cells with Ia bearing targets (Webb *et al.*, 1979; Biddison *et al.*, 1982; Krensky *et al.*, 1982; Meuer *et al.*, 1982; Rogozinski *et al.*, 1984; Gay *et al.*, 1987). Mutagenesis and epitope mapping by antibodies, have demonstrated that the two most NH_2-terminal domains of CD4 (V1 and V2) contribute to the Ia binding structure of CD4 (Clayton *et al.*, 1989; Lamarre *et al.*, 1989). The NH_2-terminal domains of CD4 also contain the binding residues most important in the interaction of CD4 with gp120, the envelope glycoprotein of human immunodeficiency virus (HIV) (Jameson *et al.*, 1988; Richardson *et al.*, 1988; Peterson and Seed, 1988; Landau *et al.*, 1988; Clayton *et al.*, 1988; Arthos *et al.*, 1989). It is the relatively high affinity interaction of the CD4 V1 domain with gp120 that renders CD4 the primary receptor for HIV entry into human cells (McDougal *et al.*, 1986*a*,*b*; Lifson *et al.*, 1986; Lasky *et al.*, 1986; Smith *et al.*, 1987; Fisher *et al.*, 1988; Deen *et al.*, 1988; Traunecker *et al.*, 1988; Hussey *et al.*, 1988). Although CD4–Ia and CD4–gp120 binding mediate cell–cell adhesion, CD4 molecules also participate in signaling events that regulate T cell activation (Bank and Chess, 1985; Moldwin *et al.*, 1987; Carrel *et al.*, 1988; Janeway, 1989). In this regard, CD4 is physically associated with a protein tyrosine kinase,

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$p56^{lck}$ and it is thought that the interaction of $p56^{lck}$ with the cytoplasmic tail of CD4 is important in signaling (Turner *et al.*, 1990).

The functional significance of the two COOH-terminal domains of CD4 (V3 and V4) has been less well studied. The monoclonal antibody (mAb) OKT4 recognizes a trypsin resistant, membrane proximal portion of the V3 domain of the CD4 molecule (Rao *et al.*, 1983). Because OKT4 does not inhibit interactions of CD4 with Ia (Rogozinski *et al.*, 1984) or gp120 (McDougal *et al.*, 1985, 1986*a*) and does not induce signals to T cells (Bank and Chess, 1985), it is not known if the V3 domain participates in distinct functions. Individuals have been identified whose CD4 molecules bind a variety of mAbs to the V1 and V2 domains of CD4 but do not bind OKT4 (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The expression of the $OKT4^-$ phenotype has been determined by family studies to be inherited in an autosomal codominant fashion (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The allele is common in people of African descent, but rare in people of other racial origins (Fuller *et al.*, 1984; Aozasa *et al.*, 1985; Stohl and Kunkel, 1984). Specifically, 8.2% of African-Americans are homozygous and 20.2% heterozygous for this $OKT4^-$ phenotype (Fuller *et al.*, 1984). In contrast, less than 0.43% of Japanese subjects are homozygous $OKT4^-$ (Aozasa *et al.*, 1985). The $OKT4^-$ allele has not been reported in Caucasians (Fuller *et al.*, 1984; Stohl and Kunkel, 1984). At the present time the structural or genetic basis for this polymorphism in the CD4 molecule is unknown.

In addition, the functional consequences of the $OKT4^-$ phenotype are not completely understood. Cells expressing CD4 molecules that are $OKT4^-$ are susceptible to HIV infection both *in vitro* and *in vivo* (Hoxie *et al.*, 1986). In general, $OKT4^-$ individuals appear to have grossly normal immune functions and are not immunosuppressed (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The $OKT4^-$ phenotype may be associated with the autoimmune disease, systemic lupus erythematosis (SLE) (Ichikawa *et al.*, 1983; Stohl and Singer, 1984; Stohl *et al.*, 1985). It has been shown that homozygous $OKT4^-$ individuals with SLE, have a functional defect in T-dependent, B cell differentiation (Stohl *et al.*, 1985). As importantly, this functional defect is observed in the unaffected (non-SLE), $OKT4^-$ siblings of $OKT4^-$ SLE patients (Stohl *et al.*, 1985). In order to elucidate the structural basis for the $OKT4^-$ phenotype and to further define functions associated with this polymorphism of the CD4 molecule, the present study identified the amino acid substitution in CD4 that confers the $OKT4^-$ phenotype.

MATERIALS AND METHODS

Cell culture

The Jurkat and Epstein–Barr virus (EBV) secreting marmoset cell lines [American Type Culture Collection (ATCC), Rockville, MD] as well as B lymphoblastoid cells (see below) were cultured in IMDM supplemented with 10% FCS. The 293 cell line (ATCC) was cultured in DMEM supplemented with 10% FCS, glutamine and essential amino acids.

Monoclonal antibodies

The mAbs; OKT4, OKT8 and W6/32 were produced by hybridomas available from the American Type Culture Collection (Rockville, MD) and purified from ascites fluid on protein A columns (Biorad, Rockville Center, NY). The mAb OKT4A was purchased from Ortho Pharmaceutical Division (Raritan, NJ).

Cytofluorographic analysis

Approximately 10^5 cells were incubated with 100 ng of the indicated mAbs for 45 min at 4°C. Cells were washed to remove unbound mAb before incubation with goat anti-mouse Ig secondary antibody coupled to fluorescein (Cappel, Cochranville, PA) and fluorescence intensity was measured on a FACSCAN Cytofluorograph (Becton-Dickinson, Mountainview, CA).

Generation of $CD4^+$ B lymphoblastoid cell lines

Peripheral blood lymphocytes were obtained from the freshly drawn blood of volunteers by centrifugation of Ficoll-Hypaque. Cells that did not rosette with sheep erythrocytes (E^-) were placed in 24-well macrotiter wells (10^6 cells/ml) and cultured with 50 μl of EBV containing supernatant from the marmoset cell line. After approximately 3 weeks, EBV transformed cultures were coated with OKT4A (100 ng/10^6 cells), washed and reacted with goat anti-mouse-IgG coated magnetic beads (40 beads/cell), according to the manufacturer's instructions (Advanced Magnetics, Cambridge, MA). Cells that bound beads were purified by magnetic sedimentation and placed back in culture. Magnetic bead selection with OKT4A was repeated at approximately 10 day intervals. After the first selection, approximately 30% of B cells bound OKT4A by FACS, after the second selection; 60–70% bound OKT4A and after three selections; 90% bound OKT4A. The cells were then cloned by limiting dilution in microtiter plates and screened by FACS for CD4 expression.

Immunoprecipitation

B lymphoblastoid cells were grown in methionine-free RPMI medium with 10% dialysed FCS before a 1 hr pulse with ^{35}S methionine (10 μC/10^6 cells) and 1.5 hr chase with FCS and subsequent lysis in ice cold 10 mM TBS buffer containing 1% NP-40, 10 μg/ml iodoacetamide and 10 μg/ml phenylmethyl sulfonyl

fluoride (PMSF). Lysates were cleared by a 30 min 14,000 rev/min spin in an eppendorf microfuge and affinity purified on a 4cc lentil lectin Sepharose 4B column (Pharmacia, Uppsala, Sweden). Eluate fractions (10^6 cpm) were precleared first by reaction with a mixture of protein A–Sepharose (Pharmacia), Sepharose CL 4B (Pharmacia) and normal mouse serum. Next, the eluates were precleared by reaction with a mixture of these bead preparations and rabbit anti-mouse IgG. Aliquots of precleared eluates were then reacted with protein A–Sepharose beads (Pharmacia) coated with 5 μg of the following murine IgG2 mAb; OKT3, OKT4, OKT4A or W6/32. The beads were washed seven times in 0.3% NP-40, 10 mM Tris and 0.5 M NaCl before addition of SDS/2-Mercapto ethanol running solution and heated to 100°C for 5 min. Samples were separated in a 10% polyacrylamide gel. The gel was fixed in 30% methanol/10% glacial acetic acid for 15 hr and treated with Autofluor (National Diagnostics, Manville, NJ) for 1 hr. After drying the gel was used to expose XAR film (Kodak, Rochester, NY).

Oligodeoxynucleotide synthesis

Oligodeoxynucleotides (oligos) were synthesized by the phosphoramidite method on either a Cyclone Plus (MilliGen/Biosearch, Burlington, MA) or a Model 381A DNA Synthesizer (Applied Biosystems, Pasadena, CA), removed from the resin by treatment with concentrated NH_4OH followed by desalting on a NAP-5 column (Pharmacia) (for oligos <40 bases in length) with H_2O elution or by the use of an OPC column (Applied Biosystems) with 20% acetonitrile elution (for oligos >40 bases in length). The numbering used throughout this paper for CD4 nucleotides and amino acids corresponds to the Maddon numbering of the CD4 gene, RWHUT4 (Maddon *et al.*, 1985) with correction (Hussey *et al.*, 1988; Littman *et al.*, 1988).

RNA polymerase chain reaction

Total RNA was isolated from 10^8 PHA stimulated E^+ cells (Chirgwin *et al.*, 1979) and cDNA was prepared by reverse transcription of approximately 1.0 μg of total RNA using 200 units of moloney murine leukemia virus (MMLV) reverse transcriptase [Bethesda Research Labs (BRL), Bethesda, MD] for 30 min at 42°C in a reaction containing 10 pM of the primer, pr1540-1517 (5′ GAT CTG CTA CAT TCA TCT GGT CCG) in 20 μl of a buffer containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 20 units of RNAsin (Pharmacia). The reaction was heated to 95°C for 5 min to inactivate the enzyme. The first strand was amplified by PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986) under the following conditions: the initial template denaturing step (8 min at 94°C), followed by a 45-fold repetitive cycle of 2 min at 55°C (annealing), 2 min at 72°C (extension) and 2 min at 94°C (denaturation) using 2.5 units DNA Taq-polymerase (Perkin-Elmer Cetus, Norwalk, CT), 200 μM each of dATP, dCTP, TTP and dGTP (Perkin-Elmer Cetus), and 50 pM of the primers pr98-117 (5′ GGC ACT TGC TTC TGG TGC TG) and pr1540-1517 in a final volume of 100 μl PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin). After amplification the samples were analyzed by electrophoresis on a 1.0% agarose gel and were stained with ethidium bromide.

Construction of $CD4^{OKT4-}$ plasmids by PCR recombination

A PCR strategy was employed to construct a $CD4^{OKT4-}$ cDNA flanked by restriction sites, suited to expression in mammalian cells. We had extensive experience expressing immunoglobulin (Ig) genes using a vector derived from pD5 (Berkner and Sharp, 1985; and see below) and a 5′ gene segment derived from Ig that encodes an Ig signal sequence (Riechmann *et al.*, 1988). A computer-based algorithm, SIGSEQ2 (Daugherty *et al.*, 1990), predicted that cleavage of the Ig signal peptide fused to CD4 would result in a normal NH_2-terminus of mature CD4 (Hussey *et al.*, 1988). The $Ig/CD4^{OKT4-}$ chimeric cDNA was generated by PCR construction of three overlapping DNA segments in separate PCR reactions, followed by assembly of the three segments into a full length cDNA using primers (amplimers) that were complementary to the 5′ and 3′ termini of the full length molecule as described in Fig. 4 (Higuchi *et al.*, 1988).

The PCR product was digested with *HindIII* and *XbaI* and ligated into pSP72 (Promega) for sequencing. In order to express the OKT4⁻ allele, a *HindIII/XbaI* insert from these pSP72 constructs were ligated into pD5-tk-hygro which contained the following segments from pD5 (Berkner and Sharp, 1985): origin of adenovirus replication, the SV40 enhancer domain, the adenovirus major late promoter, the adenovirus 2 tripartite leader, a 5′ splice donor from the adenovirus third leader and the SV40 late polyadenylation signal and in addition contained: an IgH enhancer, a 3′ splice acceptor derived from an immunoglobulin locus, a multiple cloning site (containing *HindIII* and *BamH*1) and a hygromycin resistance gene (Daugherty *et al.*, unpublished data).

Construction of CD4 plasmids

The *EcoR*1 fragment contains a full length cDNA insert from pMV7/T4 (Maddon *et al.*, 1985) encoding CD4 plus 5′ and 3′ untranslated sequence. The *EcoR*1 fragment was cloned into pCDNA-I (Invitrogen) generating $pCDNA\text{-}I/CD4^{OKT4+}$.

The plasmid, $pCDNA\text{-}I/CD4^{OKT4-}$ was generated by a three piece ligation of a 236 bp *AflII/SacI* (bp 370–606) fragment and a 265 bp *SacI/BstEII* (bp 606–871) fragment from $pSP72/CD4^{OKT4-}$ into the $pCDNA\text{-}I/CD4^{OKT4+}$ vector that had been digested with *AflII* and *BstEII*. Competent MC1061/p3 *E. coli*

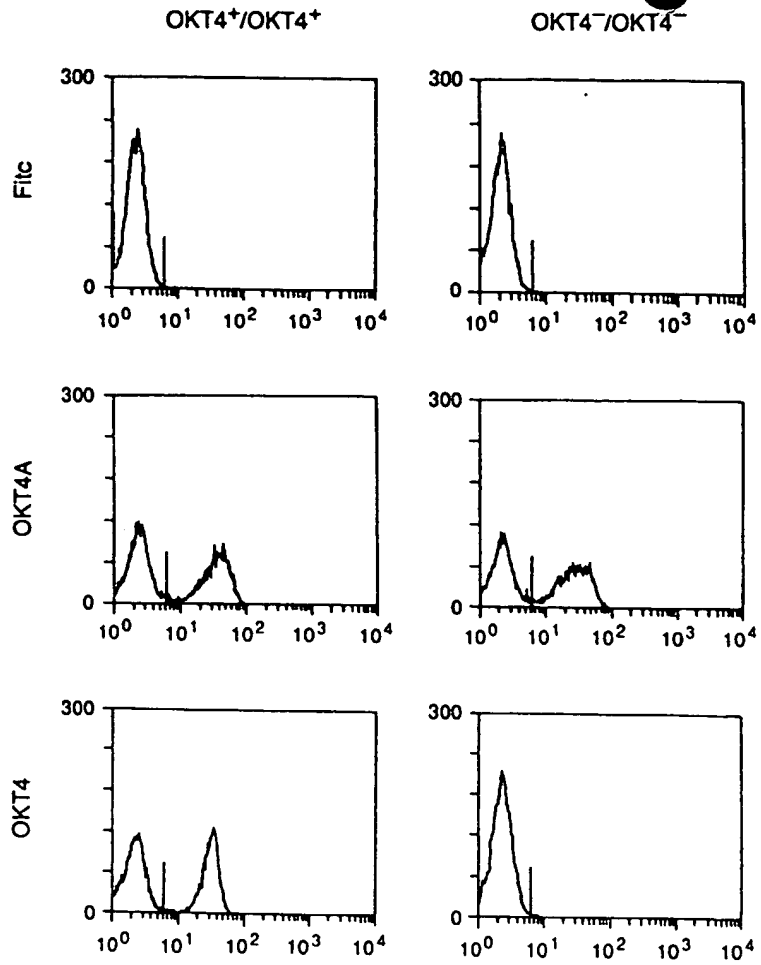

Fig. 1. FACS identification of a homozygous OKT4⁻ subject. The panels on the left are PBL from an individual with $CD4^{OKT4+}$ molecules and on the right are from the individual with $CD4^{OKT4-}$ molecules whose cells were subjected to the detailed biochemical analysis that follows. Shown are fluorescence cell histograms with the *Y* axis indicating number of cells and the *X* axis showing relative fluorescence intensity. The mAbs used are indicated on along the *Y* axis. "Fitc" designates that no primary antibody was added to cells and indicates the background fluorescence.

(Invitrogen) were transformed by the ligated DNA and grown on tetracycline (7.5 μg/ml) and ampicillin (12.5 μg/ml) plates.

Transfection of 293 cells

2×10^6 293 cells were plated on 100 mm Petri dishes 48 hr prior to transfection. The cells were fed with fresh medium 1 hr prior to transfection. Calcium phosphate precipitates were prepared (Graham and van der Eb, 1973; Pellicer *et al.*, 1978) using 20 μg of plasmid DNA per dish. After 15 hr at 37°C in 6% CO_2 the cells were fed with fresh media. Thirty-six hours after transfection, the cells were harvested by treating with trypsin-EDTA (Gibco, Grand Island, NY) for 30 sec and examined by FACS.

DNA sequencing

For sequencing of PCR products, the DNA product of RNA PCR described above was subjected to asymmetric PCR employing 50 pM of one primer and 0.5 pM of a second primer in a modification of the published protocol (Gyllensten and Erlich, 1988). Plasmid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979). Annealing oligodeoxynucleotides were 17-mers based on the sequence of $CD4^{OKT4+}$. The sequencing reactions were performed according to the manufacturer's instructions (Sequenase, Version 2.0, U.S. Biochemical, Cleveland, OH).

RESULTS

In order to facilitate the characterization of the $CD4^{OKT4-}$ protein and gene, B cells from a homozygous OKT4⁻ subject (Fig. 1) were immortalized with Epstein–Barr virus and subjected to positive selection using the mAb OKT4A and magnetic beads. Following antibody selection and limiting dilution cloning we isolated B lymphoblastoid cell clones expressing $CD4^{OKT4-}$ (Fig. 2a). Immunoprecipitation of the $CD4^{OKT4-}$ protein from $CD4^{OKT4-}$ expressing B lymphoblastoid cells using the mAb OKT4A, revealed proteins that co-migrated with OKT4⁺ CD4 proteins on SDS–PAGE electrophoresis (Fig. 2b).

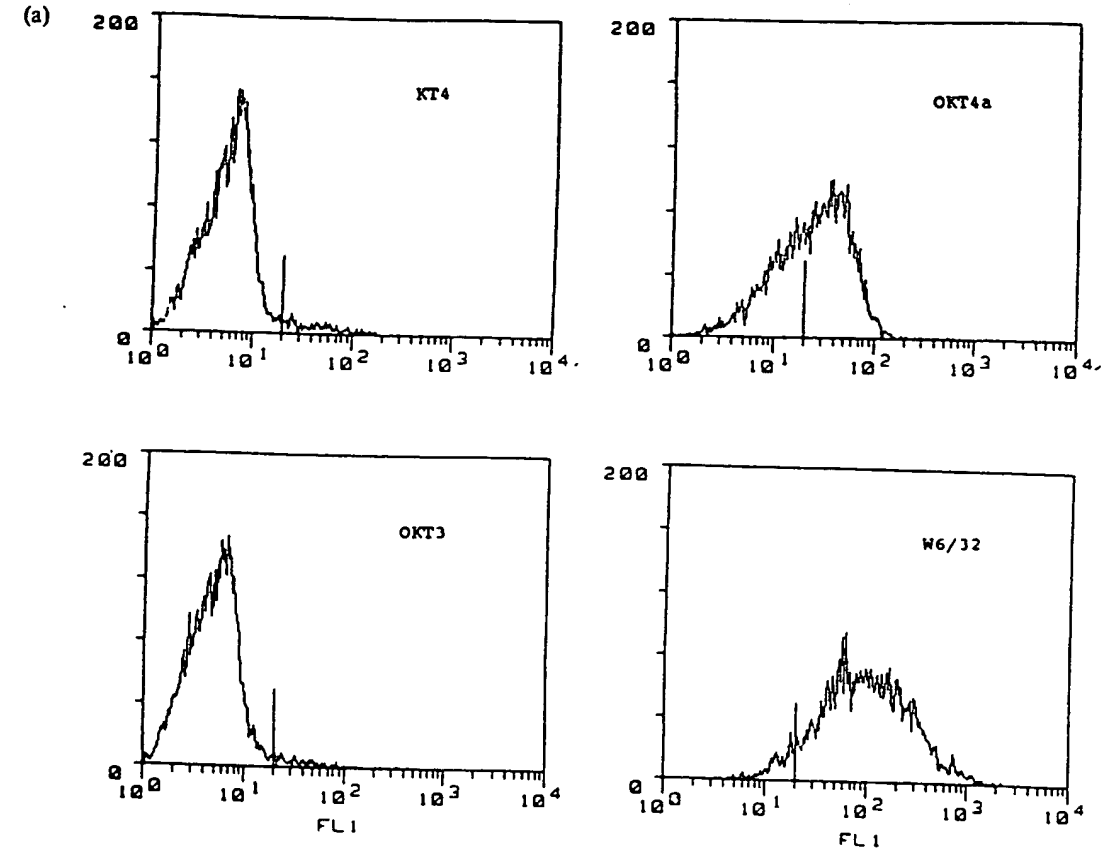

Fig. 2. Biochemical analysis of the $CD4^{OKT4-}$ protein. (a) B cell lines which express CD4 OKT4⁻. Shown is the staining of a $CD4^+$ B lymphoblastoid clone with the mAbs; OKT3 (anti-CD3), W6/32 (anti-Class I MHC) and two anti-CD4 mAbs, OKT4 and OKT4A. (b) Immunoprecipitation of $CD4^{OKT4-}$. Shown are radiographic exposures of radiolabeled proteins from a 30% $CD4^+$ B cell line, immuno-precipitated with the mAbs, OKT4A and OKT4. As a control, radiolabeled lysate from Jurkat (which is $OKT4^+$ by FACS) is also shown. The unidentified 45 kDa band bound to protein A–Sepharose beads independently of added mAb.

RNA analysis by hybridization with a CD4 probe demonstrated that the size of the message encoding the OKT4⁻ and $OKT4^+$ alleles of CD4 were indistinguishable (not shown). Taken together, the protein and Northern data suggested that the $CD4^{OKT4-}$ phenotype was not due to a large deletion in the CD4 gene.

In order to clone and sequence the cDNA encoding the $CD4^{OKT4-}$ allele, poly(A^+) RNA from PHA stimulated peripheral T cells from the homozygous OKT4⁻ subject shown in Fig. 1, was amplified by RNA PCR using oligos anchored in the 5′ and 3′ untranslated (UT) sequences of the CD4 cDNA. Next, the structure of the $CD4^{OKT4-}$ cDNA was mapped using oligos that amplified overlapping segments of the amplified CD4 template cDNA that spanned the entire CD4 coding sequence. Each of the primer pairs amplified DNA segments that

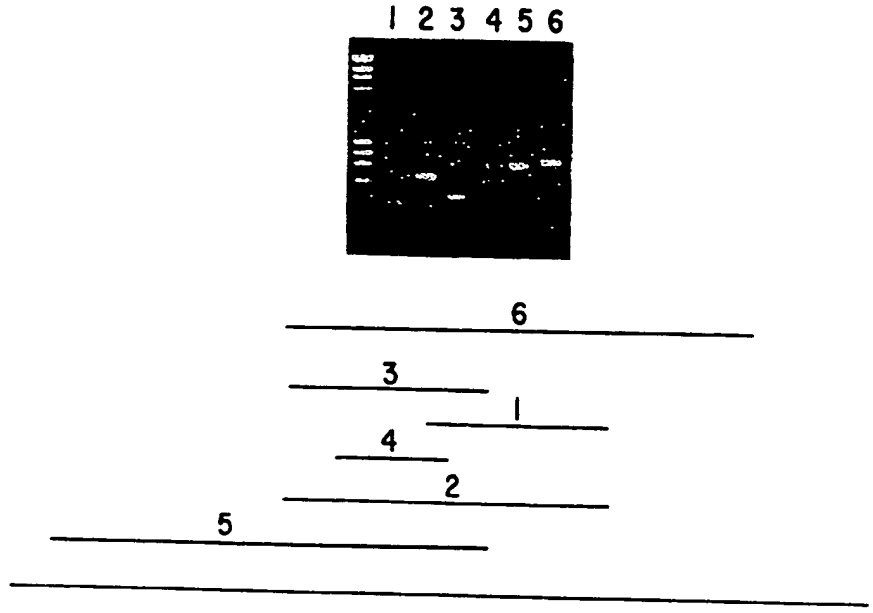

Fig. 3. Mapping the $CD4^{OKT4-}$ cDNA by PCR. First strand cDNA from T cells with the $OKT4^-$ phenotype was amplified by PCR using primer pairs based on the sequence of $CD4^{OKT4+}$ and the resulting PCR products were separated by electrophoresis in 1% agarose containing ethidium bromide and visualized by u.v. light. Lanes (1–6) represent the PCR products of the following primer pairs [the primers are identified by the nucleotide numbers represented in the primer in the 5′ to 3′ direction based on the nucleotide sequence of CD4 (1). The sequences of the primers are: pr1190–1172 (5′ AGC AGA CAC TGC CAC ATC), pr531–550 (5′ GTG CAA TGT AGG AGT CCA AG), pr 950–929 (5′ GCA TAC TGA GGC AAG GCC TG), pr617–634 (5′ CCT GGA CAT GCA CTG TC), pr892–875 (5′ TGG AGC TTA GGG TCC TG), pr97–117 (5′ GGC ACT TGC TTC TGG TGC TG), pr1450–1430 (5′ CAA ATG GGG CTA CAT GTC TTC) and pr832–849 (5′ CCT TTG ACC TGA AGA AC]: (1) pr833–849/pr1190–1173 (357 bp product), (2) pr532–551/pr1190–1173 (658 bp product); (3) pr532–551/pr950–931 (418 bp product); (4) pr617–633/pr893–877 (276 bp product); (5) pr98–117/pr950–931 (852 bp product); and (6) pr532–551/pr1451–1431 (919 bp product). The DNA standards are *λ/HindIII* (measuring 23, 9, 6.6, 4.4, 2.3 and 2.0 kb) and *ϕ/HaeIII* (measuring 1.3, 1.1, 0.9, 0.6 and 0.3 kb) (BRL).

corresponded to the expected sizes for the $OKT4^+$ allele (Fig. 3) demonstrating that the $OKT4^-$ allele did not contain a large deletion of nucleotides, relative to the $OKT4^+$ allele.

The RNA PCR products were then partially sequenced and complete sequence homology was observed between the $OKT4^-$ and $OKT4^+$ alleles in several regions including the 5′ and 3′ UT regions as well as the nucleotides encoding the NH_2- and COOH-terminal amino acids of the mature proteins. In addition, the nucleotide sequence including nucleotides 877–894, which represents an internal region, approximately midway through the coding region, was also identical. Given these regions of nucleotide sequence identity between the $OKT4^+$ and $OKT4^-$ alleles, a PCR strategy was employed to clone a gene encoding $CD4^{OKT4-}$ (Fig. 4). The PCR strategy was designed to fuse an Ig signal sequence onto a gene encoding the mature CD4 protein in order to facilitate screening of clones for expression of the phenotype (Fig. 4). Two separate PCR reactions amplified the 5′ and 3′ halves of the coding region of $CD4^{OKT4-}$ cDNA from the template of the RNA PCR product described above (Fig. 4). The resulting PCR product was subcloned in pSP72 (Promega) and four clones that were found to contain the full length recombined, CD4 cDNA were completely sequenced.

The pSP72/$CD4^{OKT4-}$ clones revealed two consistent nucleotide substitutions in four out of four clones analyzed: a C→T transition at nucleotide 352 that is silent and another C→T transition at nucleotide 867 that results in a arginine→tryptophan substitution at amino acid residue 240. Each clone contained at least two additional nucleotide substitutions, relative to the $OKT4^+$ sequence, that were not present in the other three clones or in the RNA PCR product sequenced by asymmetric PCR. These additional mutations appeared to represent random mutations introduced during the PCR amplification using the taq polymerase. Several clones were screened for expression by ligation into the pD5-tk-hygro vector and transfection into 293 cells. One clone expressed $CD4^{OKT4-}$ molecules by FACS analysis (not shown). This clone contained 867 C→T, but also contained an additional mutation (coding for an amino acid substitution in the V4 domain of the CD4 protein) that was not present in the other clones or in the sequence obtained from direct sequencing of the RNA. In order to test the hypothesis that the

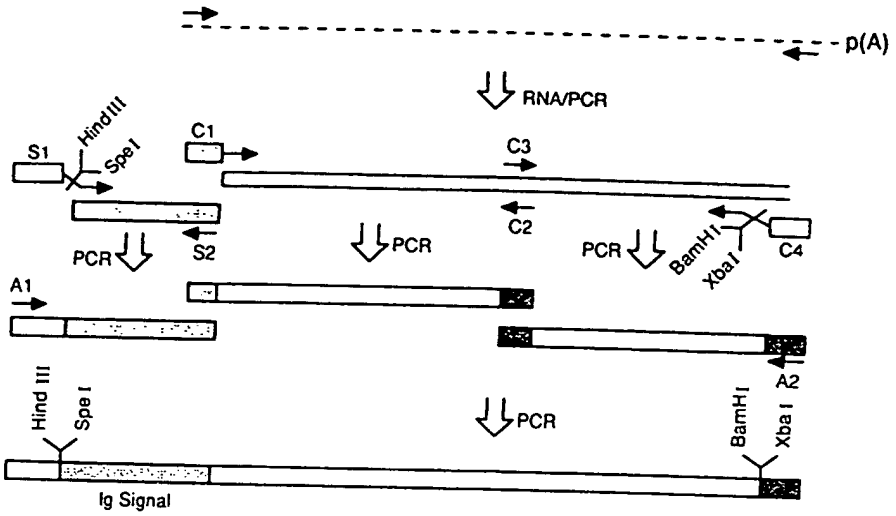

Fig. 4. Cloning of $CD4^{OKT4-}$ by PCR. The $Ig/CD4^{OKT4-}$ chimeric cDNA was generated by PCR construction of three overlapping DNA segments in separate PCR reactions, followed by assembly of the three segments into a full length cDNA using primers (amplimers) that were complementary to the 5′ and 3′ termini of the full length molecule. First, three separate PCR reactions generated: (1) a 215 bp fragment containing the Ig signal sequence (with amplimer sequence and restriction sites near the 5′ terminus); (2) a 762 bp segment of DNA encoding approximately the 5′ half (bp 151–894) of the coding region of CD4 (with a 19 bp 5′ segment of DNA overlapping the 3′ terminus of the Ig signal); and (3) a 591 bp segment encoding the 3′ half (bp 877–1449) of CD4 (with a 19 bp sequence containing amplimer sequence and restriction sites near the 3′ terminus and a 5′ segment of DNA overlapping the 3′ terminus of the 5′ half of CD4). Reaction (1) employed the primers S1 (5′ CAT TCG CTT ACC AGA TCT AAG CTT ACT AGT GAG ATC ACA GTT CTC TCT AC) and S2 (5′ GGA GTG GAC ACC TGT GGA G) and a template plasmid, NEW/M13pCR1, containing the Ig 5′ UT and signal sequence (unpublished) and generated the following DNA fragment: (5′ CAT TCG CTT ACC AGA TCT AAG CTT ACT AGT GAG ATC ACA GTT CTC TCT ACA GTT ACT GAG CAC ACA GGA CCT CAC CAT GGG ATG GAG CTG TAT CAT CCT CTT CTT GGT AGC AAC AGC TAC AGG TAA GGG GCT CAC AGT AGC AGG CTT GAG GTC TGG ACA TAT ATA TGG GTG ACA ATG ACA TCC ACT TTG CCT TTC TCT CCA CAG GTG TCC ACT CC). Primer S1 appends *HindIII* and *SpeI* restriction sites as well as an amplimer sequence at the 5′ terminus. Reactions (2) and (3) used as template, the RNA PCR product from OKT4⁻, PHA stimulated T cells shown above as the first white box. The RNA PCR is described in Materials and Methods. Reaction (2) employed the primers C1 (5′ CTC CAC AGG TGT CCA CTC CAA GAA AGT GGT GCT GGG C) and C2 (pr893–877) (5′ TGG AGC TTA GGG TCC TG). Primer C1 appends a 19 bp overlap with the Ig signal segment to the codon encoding the first amino acid of mature CD4. Reaction (3) employed the primers C3 (pr877–894) (5′ CAG GAC CCT AAG CTC CAG) and C4 (5′ GAA TGT GCC TAC TTT CTA GAG GAT CCT CAA ATG GGG CTA CAT GTC TTC). Primer C4 appends *BamH*1 and *XbaI* restriction sites as well as an amplimer sequence to the 3′ terminus of the 3′ half of the coding sequence of CD4. In each case, the PCR reactions contained 10 ng of template and 50 pM of each primer were carried out in 100 μl of PCR buffer for 25 cycles as described in Materials and Methods. The PCR products of reactions (1)–(3) were recombined using the flanking primers (amplimers), A1 (5′ CAT TCG CTT ACC AGA TCT) and A2 (5′ GAA TGT GCC TAC TTT CTA G) that were complementary to the 5′ terminus of the Ig signal segment and the 3′ terminus of the 3′ half of CD4, respectively (Fig. 4). The PCR reaction contained 10 ng of the products of reactions (1)–(3), and 50 pM of the amplimers (A1 and A2). The reaction was carried out for 25 cycles as described in Materials and Methods.

single, arginine → tryptophan substitution (867 C → T) encoded the $OKT4^-$ phenotype, a full length cDNA containing only the 867 C → T mutation, on the background of $CD4^{OKT4+}$, was generated by ligation of an internal *AflII–BstEII* fragment (bp 307–871) from $pSP72/CD4^{OKT4-}$ into $pCDNA\text{-}I/CD4^{OKT4+}$ (which includes the native CD4 signal sequence). In transient expression assays, this cDNA directed the expression of CD4 molecules with the $CD4^{OKT4-}$ phenotype (Fig. 5a). The fact that this cDNA ($pCDNA\text{-}I/CD4^{OKT4-}$) expressed a $CD4^{OKT4-}$ protein, confirmed that a single amino acid substitution of arginine for tryptophan at residue 240 in CD4 accounts for the $OKT4^-$ phenotype (Fig. 5b).

DISCUSSION

In this study we have precisely characterized the molecular basis for a common African allele of the human CD4 molecule. The allele is phenotypically characterized by the absence of reactivity of CD4 molecules with the mAB OKT4 (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). Of African-Americans studied, 20.2% express this allele as heterozygotes and 8.2% express this allele as homozygotes (Fuller *et al.*, 1984). The present study demonstrates that the genetic basis for this phenotype in one homozygous individual is a single amino acid

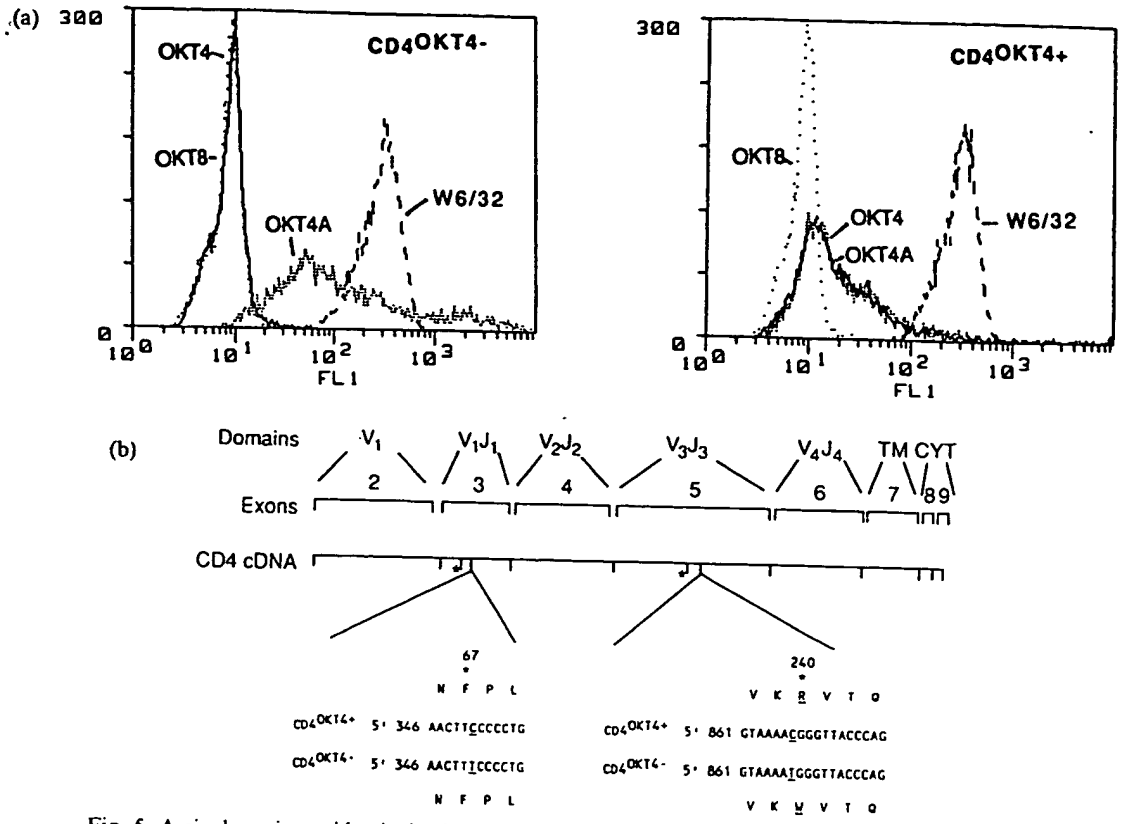

Fig. 5. A single amino acid substitution (R→W 240) encodes the CD4^{OKT4-} phenotype. (a) Expression of pCDNA-I/CD4^{OKT4-} in 293 cells. Shown are fluorescence cell histograms of 5×10^5 293 cells transfected with pCDNA-I/CD4^{OKT4-} (top panel left) or pCDNA-I/CD4^{OKT4+} (top panel right) and stained with the indicated mAbs. (b) Physical map of CD4^{OKT4-} cDNA showing relationship of nucleotide substitutions to coding changes, and exon and domain structures. Asterisks indicate potential other CD4 polymorphisms, Trp–Arg (62) and Phe–Ser (229), previously published (Fisher *et al.*,88).

substitution of tryptophan for arginine at amino acid residue 240 in the extracellular V3 domain of CD4.

Comparison of the sequence surrounding CD4 amino acid 240 with the CD4 sequence from other species, indicates that a positively charged amino acid (either arginine or lysine) is present at this position in diverse species in the primate (Camerini and Seed, 1990) and rodent (Maddon *et al.*, 1987; Clark *et al.*, 1987) orders (Fig. 6). In rat and mouse, arginine 240 is represented by a lysine that conserves the positively charged amino acid (Fig. 6). The fact that the arginine → tryptophan substitution at residue 240 does not maintain a positively charged residue at this position, suggests that a functionally important consequence may be associated with the OKT4$^-$ phenotype. However, the fact that human and other primate CD4 encodes a lysine at the nearby residue 239, may indicate redundancy in the positively charged amino acids in this region, mollifying the effects of the OKT4$^-$ substitution (Fig. 6).

Although the *in vivo* functional consequences, if any, of the CD4^{OKT4-} phenotype remains largely unexplored, it is of interest that some OKT4$^-$ individuals with the autoimmune disease, SLE, as well as their healthy (non-SLE) OKT4$^-$ relatives, demonstrate abnormal interactions between T and B cells

```
                                    240
huOKT4-                              W
human       (225) SWITFD LKNKEVSVKR VTQDPKLQMG KKLPL
rhesus      (225) ..... ..........  ..........  .....
chimpanzee  (225) ..... ..........  ..........  .....
mouse       (224) P..S.S I.......QK S.K.L...LQ ET...
rat         (224) .....S ...QK...QK S.SNP.F.LS ET...
poly(Ig)R   (157) --LII. SSS..AKDP. YKGRIT..IQ STTAK
```

Fig. 6. Comparison of the CD4 sequence in the region of the OKT4$^-$ mutation with other related proteins. The human sequence in this region is completely conserved between chimpanzee, rhesus macaque (Camerini and Seed, 1990). The mouse (Maddon *et al.*, 1987) and rat (Clark *et al.*, 1987) CD4 proteins have a lysine at the position equivalent to human residue 240. The comparison of the CD4 sequence to that of the rat poly Ig receptor is published (Clark *et al.*, 1987) and indicates that the poly Ig receptor contains an arginine at the residue equivalent to CD4 240. Dots indicate identity with the human sequence and dashes represent gaps in the amino acid sequence used to maximize homology (Clark *et al.*, 1987).

in the generation of antibody responses *in vitro* (Stohl *et al.*, 1985). Because the mutation accounting for the $CD4^{OKT4-}$ phenotype resides in V3 and not in the Ia binding regions of CD4, any functional consequences of the mutation, such as the reported abnormalities in T–B interactions (Stohl *et al.*, 1985), would most likely be a consequence of CD4 functions unrelated to Ia binding. In this regard, there is evidence that CD4 may physically interact in a functionally important manner with the antigen specific T cell receptor (Saizawa *et al.*, 1987; Anderson *et al.*, 1988; Rojo *et al.*, 1989; Rivas *et al.*, 1988; Mittler *et al.*, 1989). The molecular basis for this putative interaction is unknown but it is of interest that the location of the arginine to tryptophan substitution in the membrane proximal, V3 domain of CD4, suggests a region of the molecule that might interact horizontally with a ligand confined to the same membrane, such as the T cell receptor. Efforts are underway to express the $OKT4^-$ allele as well as the $OKT4^+$ allele in the context of a functional TCR on the surface of a $CD4^-$ T cell clone in order to investigate this point in detail.

We and others have previously reported that IgG binds to recombinant truncated CD4 (Lederman *et al.*, 1990; Lenert *et al.*, 1990). The precise site on CD4 that binds IgG is controversial, but we have provided evidence suggesting that at least one site of IgG interaction with CD4 is either the V3 or V4 region of the soluble CD4 molecule (Lederman *et al.*, 1990). Although we cannot detect IgG binding to cellular CD4 molecules (Lederman *et al.*, unpublished), and the physiological significance of CD4–IgG binding is not known, the fact that IgG may bind to a site near to the present amino acid substitution is interesting. In this regard, it may be important to note that the poly Ig receptor contains a domain that is homologous to CD4 V3 and has an arginine at the amino acid residue analogous to the arginine at 240 of CD4 (Clark *et al.*, 1987). Although there is no direct evidence that T cells interact with IgG molecules during T–B collaboration, the observation that aggregated IgG inhibited T–B interactions is consistent with such an interaction (Nicholson and McDougal, 1981).

Binding studies using anti-CD4 mAbs have detected other polymorphisms, but the incidence of these alleles has not been studied in populations. For example, a polymorphism was detected in an $OKT4^-$ subject that was deficient in the binding of the mAb OKT4C (Bach *et al.*, 1981). In addition, a subject was recently reported to be deficient in the binding of the mAb Leu3a (Angadi, 1990). Both OKT4C and Leu3a bind to amino acid residues in the Vl domain of CD4 (Jameson *et al.*, 1988). Furthermore, two other nucleotide changes that encode amino acid substitutions were identified by the independent isolation of a clone encoding CD4 from a T cell cDNA library (Fisher *et al.*, 1988). Interestingly, these polymorphisms are encoded by sites on the CD4 gene close to the positions where nucleotide changes are found in the $CD4^{OKT4-}$ allele analyzed in the present work (Fig. 6). In that study no correlation was made between the genetic structure and the phenotype of the encoded protein in relation to the binding of mAbs. Therefore, it appears that several polymorphic alleles of CD4 exist and the limited genetic data suggest that mutations are clustered into two areas of the protein, encoded by different exons (Fig. 5b) (Maddon *et al.*, 1987). It is not clear if these clusters indicate areas of active mutation or if there are selective functional pressures exerted on these regions. In this regard, it is interesting that analysis of the CD4 protein sequence suggests that amino acids 62, 229 and 240 may be located on alpha helices (Maddon *et al.*, 1987) which often form turns on the external surface of the protein.

It is interesting to note that a high prevalence of the $OKT4^-$ allele coexists in Africa with a relatively high prevalence of human infection by the CD4 trophic pathogenic human retroviruses (HIV-1 and -2). The infectibility of $CD4^{OKT4-}$ cells by HIV in culture is established (Hoxie *et al.*, 1986), but a potentially important avenue of future study is to determine if the CD4 polymorphism characterized in the present study affects the clinical outcome of HIV infection. If this were the case, molecular analysis of the mechanism of such an effect would be potentiated by the genetic tools described here.

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Exhibit D

Effects of Amino Acid Substitutions Outside an Antigenic Site on Protein Binding to Monoclonal Antibodies of Predetermined Specificity Obtained by Peptide Immunization: Demonstration with Region 94–100 (Antigenic Site 3) of Myoglobin

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Amino acid substitutions outside protein antigenic sites are very frequently assumed to exert no effect on binding to antiprotein antibodies, especially if these are monoclonal antibodies (mAbs). In fact, a very popular method for localization of residues in protein antigenic sites is based on the interpretation that whenever a replacement causes a change in binding to antibody, then that residue will be located in the antigenic site. To test this assumption, mAbs of predetermined specificity were prepared by immunization with a free (i.e., without coupling to any carrier) synthetic peptide representing region 94–100 of sperm whale myoglobin (Mb). The cross-reactivities and relative affinities of three mAbs with eight Mb variants were studied. Five Mb variants which had no substitutions within the boundaries of the designed antigenic site exhibited remarkable, and in two cases almost complete, loss in cross-reactivity relative to the reference antigen, sperm whale Mb. Two myoglobins, each of which had one substitution within region 94–100, showed little or no reactivity with the three mAbs. It is concluded that substitutions outside an antigenic site can exert drastic effects on the reactivity of a protein with mAbs against the site and that caution should be exercised in interpreting cross-reactivity data of proteins to implicate residues directly in an antigenic site.

KEY WORDS: Amino acid substitutions; monoclonal antibodies; myoglobin; predetermined specificity; synthetic antigenic site.

1. INTRODUCTION[3]

Immunochemical cross-reactions of protein mutants are very often used to implicate the involvement of certain amino acid residues on a protein in its antigenic sites. The data are interpreted on the basis of the assumption that every amino acid replacement on the antigen which results in changes in its binding properties is directly involved in the interaction with antibody, particularly when a monoclonal antibody (mAb) is used (Berzofsky *et al.*, 1980, 1982, 1983; van Regenmortel, 1988). On the other hand, it has been shown that substitutions outside protein antigenic sites will influence the antigenic reactivity of the site because of one or more of the following factors: conformational and steric readjustments, electron delocalization, charge redistribution, and changes in hydrogen bonding or hydrophobic interactions (Kazim and Atassi, 1980; Atassi and Kazim, 1980;

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[3] Abbreviations used: BSA, bovine serum albumin; mAb, monoclonal antibody; mAb C_{50}, monoclonal antibody concentration at 50% cross-reactivity; Mb, myoglobin and when preceded by an abbreviation it denotes the following Species: Bg, badger, Ch, chicken; Cs, California sea lion; Dg, dog; Ed, echidna; Fb, finback whale; Pd; pacific common dolphin; Sp, Sperm whale; PBS, 0.15 M Nacl in 0.01 M sodium phosphate buffer, pH 7.2; RIA, radioimmune assay.

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Twining *et al.*, 1981). These latter studies were done with polyclonal antibodies whose recognition was directed at a number of antigenic sites in each multideterminant protein antigen [five in Mb (Atassi, 1975) and three in lysozyme (Atassi, 1987)]. Therefore, the assignment of the effects of substitutions on a given site was difficult to determine and could only be estimated from their distances in the three-dimensional (3D) structure from the site residues. Clearly, to resolve this issue, mAbs against a single site are needed whose specificity is known precisely and which is not deduced by the very approach it is set out to test.

To determine in an unequivocal manner whether the reactivity of an antigenic site is influenced by substitutions outside the site, we have employed here monoclonal antibodies that were generated by immunization with a synthetic peptide representing region 94–100 [antigenic site 3 (Atassi, 1975)] of SpMb. The peptide was used as an immunogen in its free state (i.e., without coupling to any carrier) to produce mAbs of predetermined specificity (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; for review, see Atassi and Young, 1985; Atassi, 1986). Cross-reactions of Mbs which do not have substitutions in this region revealed that substitutions outside the site can exert dramatic effects on the binding of a protein with anti-site mAbs.

2. MATERIALS AND METHODS

2.1. Materials

The isolation and purification of myoglobins of various species were performed by previously reported procedures (Atassi, 1964, 1970; Twining *et al.*, 1980). BgMb, CsMb, and PdMb were obtained from Dr. C. R. Young (Texas A&M University). The homogeneity of each Mb was confirmed by polyacrylamide-gel electrophoresis. The synthetic peptide, Ala-Thr-Lys-His-Lys-Ile-Pro, corresponds to antigenic site 3 (residues 94–100) of SpMb (Atassi, 1975). Synthesis, purification, and characterization of the peptide have been described previously (Pai and Atassi, 1975). The synthetic peptide was used as an immunogen *in its form* (i.e., without coupling to any carrier) in Balb By J mice (6–8 weeks). The mice were injected and boosted biweekly with 25 μg of peptide [previously found (Young *et al.*, 1983) to be the optimum dose in this strain] as an emulsion of a solution in PBS (50 μl) with an equal volume of Freund's complete adjuvant. The mice received boosters until good antibody titers against the peptide and SpMb were obtained in the sera. Monoclonal antibodies were prepared as described (Schmitz *et al.*, 1982, 1983*a*, *b*). Sera and culture supernatants were screened for hybridoma antibodies by a solid-phase RIA on poly(vinylchloride) plates (Costar, Cambridge, Massachusetts) using the peptide-BSA conjugate and SpMb as the plate antigens (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*).

2.2. Determination of PVC-Plates Capacity for Various Myoglobins

It was necessary to rule out the possibility that any differences that might be found in the binding of mAbs to the various Mbs will not be caused by differences in the amounts of Mbs bound to the plates. Therefore, the amount of each Mb required to saturate the plate was determined. Each protein (25 μg) was labeled with ^{125}I by the chloramine-T method (Hunter, 1969). A fixed amount of each Mb (5×10^5 cpm) was mixed with the respective unlabeled Mb (25 μg). Serial dilutions were made and 50 μl aliquots of each dilution were plated in triplicate into the wells of the plate. The plates were incubated at 37°C for 3 hr, after which they were washed six times with PBS, dried, cut out, and counted in gamma counter. The amount of each labeled Mb bound (in cpm) was plotted against its concentration. From these plots, it was possible to determine, for each Mb, the concentration needed to saturate the well.

2.3. Determination of Cross-Reactivities and Relative Affinities

Cross-reactivity studies were done as described by East *et al.*, (1982) and Leach (1983). In the present work, pvc plates were incubated (37°C, 3 hr) with saturating amounts (50 μl of 50 μg/ml) of various myoglobins after which they were blocked (37°C, 1 hr) with 100 μl of 1% BSA in PBS. Aliquots (50 μl) of serial dilutions of each mAb (in 0.1% BSA in PBS) were added to the wells and the plates were incubated at room temperature for 5 hr and then washed (five times) with PBS. The second antibody (rabbit anti-mouse IgMk), appropriately diluted with PBS-0.1 BSA, was added (50 μl) and the plates were incubated at 37°C for 2 hr. The plates were washed six times with PBS and then ^{125}I-labeled protein A (2×10^5 cpm) was added in 50 μl of PBS-0.1% BSA. Following incubation at room temperatue for 2 h, the plates were

washed with PBS and dried. The wells were then cut out and their radioactivity measured in a gamma counter. Nonspecific binding was calculated from the binding of antipeptide mAbs (the test antibodies) to wells coated with BSA. Additional negative controls included the binding, to each test Mb, of normal mouse and human IgM and culture supernatant from P3 × 63-Ag8-653 myeloma cell lines. The results were expressed in percent cross-reactivity relative to antibody bound to SpMb as 100%. Measurements of relative binding affinities were determined by antibody dilution analysis (van Heyningen, 1986). Serial dilutions of mAb were allowed to bind to each Mb as described above. Affinities were ranked on the basis of the concentration of antibody required to give 50% of maximum binding (C_{50}) relative to the C_{50} with SpMb as 1.00. This relative ranking of affinities is valid only for mAbs which bind to a single determinant on the antigen used for dilution analysis, which is the case here. Concentrations of mAb solutions were determined by calibrated solid-phase RIA relative to standard curves of known concentrations of affinity-purified mouse IgMk.

2.4. Calculation of the Distance between Site Residues and All the Other Mb Residues

The C^{α}-to-C^{α} distances as well as the atoms of nearest approach between each of the residues 94 through 100 (antigenic site 3) of SpMb and all other residues in the molecule were calculated, from the SpMb coordinates at 2.0 Å (Takano, 1984), on a VAX 8550 using the program chain (Sacks, 1988). These distances had previously been calculated (Kazim and Atassi, 1980) but subsequent refinement of Mb structure (Takano, 1984) necessitated the recalculation of these distances.

3. RESULTS

3.1. Binding of the Myoglobins to the Plates

It was necessary to ensure at the outset that the differences in the antigenic reactivities of the various Mbs were not caused by differences in the amounts of each Mb bound to the plates. The results (Fig. 1) using ^{125}I-labeled Mbs showed that each Mb was able to saturate the well when its concentration was 25 μg/ml (1.5 μg/well). Therefore, in all subsequent studies, the Mb solutions employed 50μg/ml (2.5 μg/well), which is twice the concentration necessary to saturate the wells.

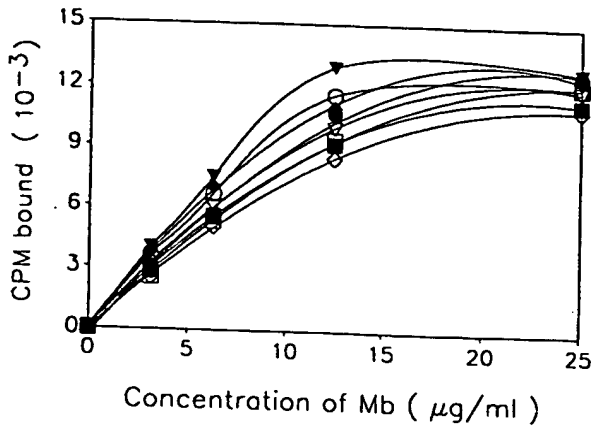

Fig. 1. Determination of PVC-plate binding capacity to myoglobin variants. The binding capacity of the wells of PVC plates for the various myoglobins was determined using increased amounts of ^{125}I-labeled Mb and unlabeled Mb: (○) BgMb, (■) ChMb, (◆) CsMb, (▼) DgMb, (▽) EdMb, (◇) FbMb, (□) PdMb, and ( ) SpMb. For experimental details, see the text.

3.2. Characterization of Antipeptide Antibodies

Pre-immune sera from the mice showed no antibody binding to the immunizing peptide 94–100 or to SpMb. The mice were injected with the optimum dose (25 μg/mouse) of peptide 94–100 and boosted until the sera possessed high titers of antibodies that bound to the peptide and to SpMb. The polyclonal antisera and the mAbs obtained from the mouse B cells after hybridization were entirely specific to the peptide and SpMb. They did not react with any SpMb peptides or any other proteins and peptides (from our large library of synthetic peptides) that are not related to the Mb system. The specificity of peptide-generated mAbs was in agreement with previous reports (Schmitz *et al.*, 1983*a*, *b*; Atassi and Young, 1985).

A panel of 18 mAbs giving high levels of binding to SpMb was prepared from fusion of spleen cells (6 mAbs) and of lymph node cells (12 mAbs). All the mAbs (as well as the polyclonal antisera) were of IgM class and *k* light chain. Three mAbs (nos. 708, 718, and 720) were used for this work on the basis of their higher relative-binding affinities

3.3. Binding of the Monoclonal Antibodies to the Various Myoglobins

Of the myoglobins prepared for those studies, five have no substitutions within the region 94–100 (to which the antibody specificity is directed) and two have each one substitution in the site (Table I).

Table I. Antigenic Site 3 of Sperm Whale Mb and Substitutions Within This Region in Other Myoglobins[a]

Myoglobin	Residue no.	94	Site 3 95	96	97	98	99	100
Sperm whale		Ala	Thr	Lys	His	Lys	Ile	Pro
Finback whale								
Pacific common dolphin								
California sea lion								
Dog								
Chicken								
Echidna								Ser
Badger							Val	

[a] The amino acid substitutions were based on the sequences given in the references cited: sperm whale Mb (Edmundson, 1965; Romero-Herrera and Lehmann, 1974); finback whale Mb (DiMarchi *et al.*, 1978); Pacific common dolphin Mb (Wang *et al.*, 1977); California sea lion Mb (Vigna *et al.*, 1974); dog Mb (Dumur *et al.*, 1976); chicken Mb (DeConinck *et al.*, 1975; Romero-Herrera *et al.*, 1978); echidna Mb (Castillo *et al.*, 1978), and badger Mb (Tetaert *et al.*, 1974).

The myoglobins that have no substitutions within region 94–100 are CsMb, FbMb, PdMb, ChMb, and DgMb. The cross-reactions of these myoglobins with antipeptide 94–100 mAbs 708, 718, and 720 were determined as a function of mAbs concentration. The results of binding to CsMb, FbMb, and PdMb are shown in Fig. 2, while Fig. 3 summarizes the results with ChMb and DgMb. With a given mAb, the cross-reactivity of each of these five myoglobins was markedly decreased relative to the reference antigen, SpMb. The extents of maximum cross-reactions (relative to SpMb as 100%) of CsMb, FbMb, and PdMb with mAbs 708, 718, and 720 in that order were CsMb, 47%, 35%, and 43%; FbMb, 47%, 37%, and 64%; PdMb, 39%, 40%, and 61%. In the case of ChMb and DgMb, their cross-reactions were dramatically and unexpectedly low (less than 20%, Fig. 3).

For the two myoglobins of eschidna and badger, each has one substitution within region 94–100 (Table I). These two myoglobins showed little or no reactivity with the three mAbs (Fig. 4).

Expression of the results in terms of the relative-binding affinities (Fig. 5) showed that all seven myoglobins (including the five that have no substitutions within the boundaries of the site) had greatly reduced binding affinities, relative to SpMb. With only one mAb (no. 720), two myoglobins that have no substitutions in the site (PdMb and FbMb) showed relative affinities that were measurable by this method, and even these were much lower (70%) than that of SpMb with this mAb.

4. DISCUSSION

It was found relatively early that the reaction of a protein antigen with its antibodies is influenced by conformational changes (Atassi, 1967, 1970; Habeeb and Atassi, 1971). The first clear evidence of this was in fact obtained with Mb and hemoglobin. In these two proteins, even though the heme group is not part of an antigenic site (Reichlin *et al.*, 1963; Atassi, 1967), derivatives whose conformation was intentionally altered using hemes with modified side chains or metal substitutions (to replace the iron) exhibited remarkable changes in antigenic reactivity (Atassi, 1967; Atassi and Skalski, 1969; Andres and Atassi, 1970). Subsequent findings with other protein systems (Atassi *et al.*, 1970; Habeeb and Atassi, 1971; Arnon and Maron, 1971, Arnheim, 1973; Prager *et al.*, 1974; Atassi and Habeeb, 1977; Habeeb, 1977) made it evident that the antibody response against native protein antigens is directed against their native three-dimensional structure (Atassi, 1967, 1975, 1978, 1984). This is now a well-established principle in molecular immunology.

Amino acid substitutions can cause conformational readjustments which will influence antigenic reactivity (Atassi, 1970; Atassi *et al.*, 1970; Arnheim, 1973, Prager *et al.*, 1974). Quite often, however, amino acid substitutions do not induce any detectable conformational changes but will nevertheless influence antigenic reactivity. The replacements may result in electron delocalization and charge redistribution and/or changes of hydrogen bonding and hydrophobic interactions which could alter the binding properties of an antigenic site. Indeed, studies with Mb (Kazim and Atassi, 1980; Twining *et al.*, 1980) and lysozyme (Atassi and Kazim, 1980) showed that substitutions outside the antigenic sites of the respective protein (Atassi, 1975, 1978) exerted remarkable effects on its antigenic reactivity, and it was concluded that not every replacement that causes a change in

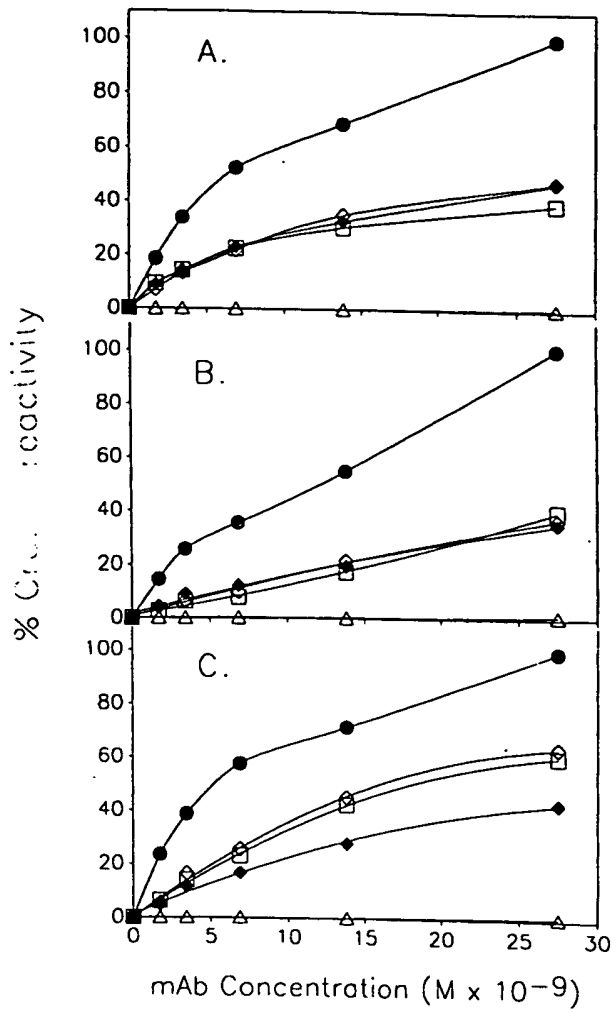

Fig. 2. Cross-reactivity of antisite 3 mAbs with PDMb, FbMb, and CsMb, which have no substitutions in this site. This binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), PdMb (□), FbMb (◇), and CsMb (◆) was determined. The microtiter wells were coated with 50 μl of Mb solutions having the same protein concentration (50 μg/ml) and the assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, PdMb, FbMb, and CsMb.

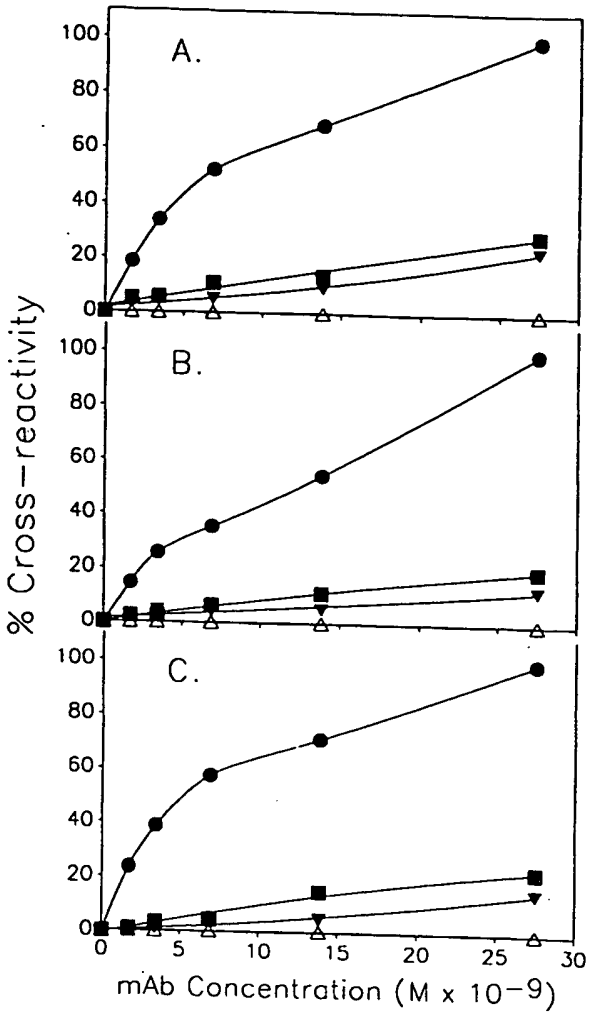

Fig. 3. Cross-reactivity of antisite 3 mAbs with ChMb and DgMb which have no substitution in that site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentration, to SpMb (●), ChMb (■), and DgMb (▼) was determined. The microtiter wells were coated with (50 μl of Mb solutions having the same antigen concentration (50 μg/ml)). The assays were done as described under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to the maximum bound by SpMb) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3 × 63-Ag8-653 myeloma cell line) to SpMb, ChMb, and DgMb.

antigenic reactivity will necessarily reside in an antigenic site.

On the other hand, others have preferred to ascribe a direct role for all replacements that influence reaction of antigen with antibody, particularly when the latter is a monoclonal antibody. Thus, it has been clearly stated that "amino acid substitutions which affect binding by monoclonal antibodies are most likely to be within the antigenic site than far from it" (Berzofsky *et al.*, 1982). More recently, it was similarly

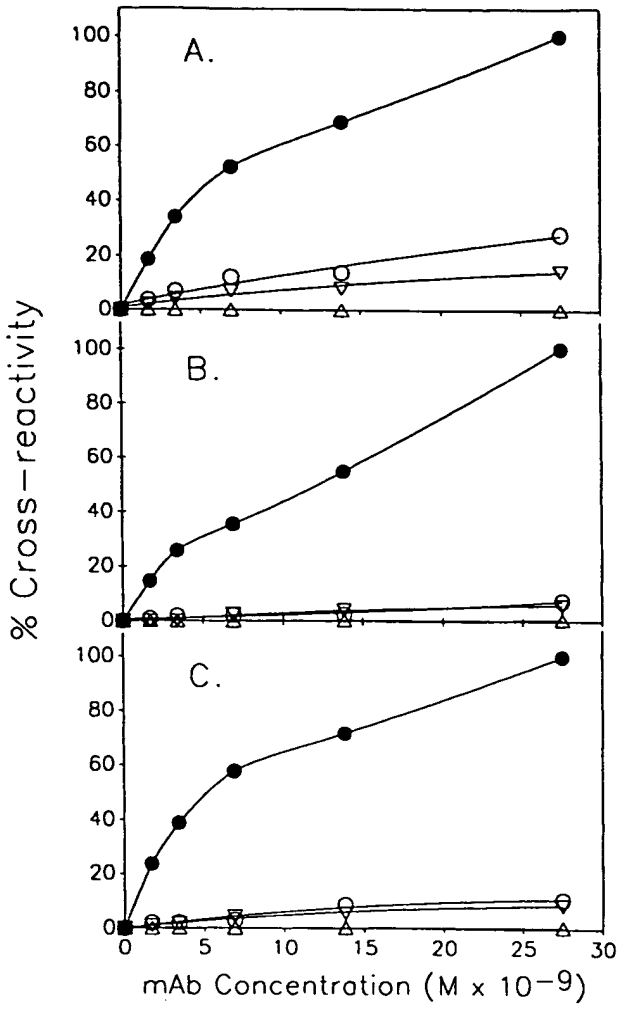

Fig. 4. Cross-reactivity of antisite 3 mAbs with EdMb and BgMb which have substitutions in and outside the site. The binding of various concentrations of antisite 3 mAbs no. 708 (A), 718 (B), and 720 (C), preadjusted to the same antibody concentrations to microtiter wells coated with 50 μl of SpMb (●), EdMb (▽), and BgMb (○) having the same antigen concentration (50 μg/ml) was done as shown under *Materials and Methods*. The values were corrected for nonspecific binding to BSA (400–1500 cpm) and the corrected values are plotted as a percentage cross-reactivity (relative to maximum bound by SpMb as 100%) at different concentrations [M] of mAb. The cpm values of 100% cross-reactivities were: 17,390 cpm, 11,967 cpm, and 14,014 cpm for mAbs no. 708, 718, and 720, respectively. The open triangle represents the binding of other negative controls (normal mouse and human IgM and culture supernatant of P3×63-Ag8-653 myeloma cell line) to SpMb, EdMb, and DgMb.

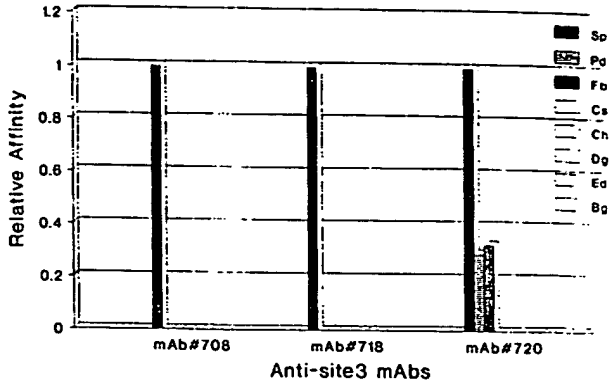

Fig. 5. Relative binding affinities of antisite 3 mAbs to myoglobin variants. The relative binding of antisite 3 mAbs to each myoglobin is calculated from mAb concentrations at 50% cross-reactivity relative to SpMb "the reference antigen" as 1.0. The relative binding affinities of mAbs 708 and 718 (to PdMb, FbMb, CsMb, ChMb, DgMb, EdMb, and BgMb) and of mAb 720 (to CsMb, ChMb, DgMb, and BgMb) could not be obtained because their maximum cross-reactivities were <50% (see Figs. 2-4).

stated that "if the substitution leads to a change in antibody binding, the mutated residue is likely to be directly involved in the structure of an epitope". And this method gives unambiguous information only when monoclonal antibodies are used as probes and the tertiary structure of the protein is known" (van Regenmortel, 1988). Based on this method of interpretation, a large number of protein antigenic sites have been described (e.g., Reichlin, 1972; Urbanski and Margoliash, 1977; Reichlin and Eng, 1978; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Hannum and Margoliash, 1985; Berzofsky and Berkower, 1989).

The findings mentioned above—which showed (Kazim and Atassi, 1980; Atassi and Kazim, 1980; Twining *et al.*, 1980) that substitutions outside the known antigenic sites of Mb (Atassi, 1975) and lysozyme (Atassi, 1978) influenced the antigenic reactivity of each protein with its respective antibodies—were done with polyclonal antiprotein antibodies. The specificities of these antibodies had been mapped exhaustively by a number of independent chemical, synthetic, and immunochemical approaches (Atassi, 1975, 1978). Because the polyclonal antibodies were capable of binding to a number of antigenic sites (five in Mb and three in lysozyme), the effects of substitutions on the binding properties of a given antigenic site were difficult to isolate and were estimated from the distances of the replacements from site residue (Kazim and Atassi, 1980; Twining *et al.*, 1980). A simpler system was therefore required to examine in an unequivocal manner the effect of substitutions outside an antigenic site whose boundaries are known precisely. This would also resolve whether cross-reactivities of protein mutants is a reliable approach for localization of antigenic sites. Clearly, to investigate

Table II. Nearest-Neighbour Residues to Antigenic Site 3 of Sperm Whale Mb[a]

Nearest neighbor residue	Minimum distances and closest atoms to residues in antigenic site 3						
	Ala-94	Thr-95	Lys-96	His-97	Lys-98	Ile-99	Pro-100
Lys-42					4.43 NZ-O	5.29 CG-CD_1	4.48 CE-CD
Phe-43						6.53 CE_2-CD_1	
Ala-90	5.70 O-N						
Ser-92			6.94 O-N	4.22 O-N			
His-93				4.80 O-C		5.75 O-C	
Ala-94		3.95 C-N					
Thr-95	3.94 N-C						
Lys-96				6.66 C-N			
His-97			6.66 N-C			6.79 C-N	
Ile-99				6.79 N-C			5.04 C-N
Pro-100						5.04 N-N	
Tyr-103							4.92 N-O
Leu-104						6.89 CD_2-CG_2	
Tyr-146	5.01 OH-N					6.26 OH-O	6.40 CE_1-C
Leu-149	5.85 CD_1-CB	5.01 CD_1-CG_2					
Tyr-151	5.78 CE_1-O	4.24 CE_2-CG_2					

[a] The first atom notation refers to the neighboring residue and the second atom refers to the site residue (for atom notations, see Watson, 1969). The distances of the nearest-neighbor residues from site 3 residues were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

these questions, mAbs are needed whose specificities are predetermined by the investigator and thus are precisely known. This was made possible by the discovery that small peptides (six residues or longer) can be used as immunogens in their free form (i.e., without coupling to any carrier) to generate polyclonal and mAbs of predetermined specificities (Young and Atassi, 1982; Schmitz *et al.*, 1982, 1983*a*, *b*; Young *et al.*, 1983; for review, see Atassi, 1986). In this study a free synthetic peptide was employed as the immunizing antigen. Since a carrier was not used, no additional unknown residues (from the carrier) would contribute to binding with antibodies against the peptide. Although, clearly, any protein whose covalent and 3D structures are known could be employed as a model for such studies, we have used here SpMb, because we have already made a large number of synthetic Mb peptides and have isolated a number of myoglobins from various species. In addition, these studies will afford the opportunity to check whether it has discontinuous antigenic sites. These had been surmised to exist in Mb entirely from interpretation of cross-reaction results based on the aforementioned assumption (i.e., that replacements causing a change are part of the site).

The nearest neighbor residues of antigenic site 3 (residues 94–100), which represent the immediate (within 7.0 Å) molecular environment of the site are listed in Table II. The replacements in these nearest-neighbor residues in the Mb variants used in this study are given in Table III. The Mb variants (Table I) were selected based on the substitutions within the region 94–100. Some variants had no substitutions in that region while others have substitutions both inside and

Table III. Nearest-neighbor (Environmental) Residues to Antigenic Site 3 of Sperm Whale Mb That Undergo Substitution in Other Myoglobins and the Nature of Substitutions[a]

Antigenic site 3 environment in sperm whale Mb	Environmental residue substitutions in other myoglobins						
	PdMb	FbMb	CsMb	ChMb	DgMb	EdMb	BqMb
Lys-42				Arg			
Phe-3							
Ala-90							
Ser-92							
His-93							
Ala-94							
Thr-95							
Lys-96							
His-97							
Ile-99							
Pro-100							Val
Tyr-103						Ser	
Leu-104						Phe	
Tyr-146							
Leu-149				Phe		Phe	
Tyr-151	Phe	Phe	Phe	Phe	Phe	Phe	Phe

[a] For the distances and molecular contacts of environmental residues within 7 Å from a site residue, see Table II. Distances were calculated from the 2.0 Å coordinates of the SpMb structure (Takano, 1984).

outside the region. The Mb variants will, therefore, be divided according to these substitutions and will be discussed below.

The finding here, that PdMb, FbMb and CsMb, which have no substitutions within the region 94–100 (Table I), exhibited much lower reactivities and relative binding affinities to antisite 3 mAbs than the reference antigen, SpMb, clearly demonstrate that amino acid substitutions outside the region 94–100 can extend their influences to alter the reactivity of that region. If the region 94–100, to which the specificity of these mAbs is directed is not affected by changes elsewhere in the molecule then the cross-reactivities of each of the three myoglobins should be equal to SpMb. The amino acid replacements which are located outside antigenic site 3 and must be responsible for these changes are listed inTable IV, together with their minimum distances from the site (which ranged from 4.24–34.86 Å). Another pair of Mb variants (ChMb and DgMb), which also have no substitutions in site 3 (Table I), exhibited drastic losses in their cross-reactivities with antisite 3 mAbs (Figs. 3 and 5). These dramatic immunochemical changes obviously stemmed from effects exerted by amino acid substitutions outside the region 94–100. These substitutions are listed in Table V, together with their minimum distances from the site which varied from 4.22–33.76 Å.

Of the echidna and badger Mbs, each has a single substitution in site 3 (Table I), in addition to the substitutions elsewhere in the molecule. In this case, will be uncertain whether to attribute the complete destruction in their cross-reactivities to the single amino acid substitution within the site or to those outside the antigenic site. It is quite likely the result of both. The minimum distances of these substitutions (which varied from 4.24–33.76 Å, from site 3) are given in Table VI.

The observed changes in the immunochemical reactivity of a given Mb variant, relative to SpMb were most likely due to the cumulative indirect effects of amino acid substitutions outside site 3. The individual contribution of each substitution to the overall effect is difficult to determine but would be a function of the nature of the replacement and its distance from the antigenic site. Further, it should be expected that the extent of influences in a given Mb of amino acid substitutions outside the site is also dependent on the number of amino acid substitutions. This was clear in the binding properties of PdMb, FbMb, and CsMb compared to ChMb and DgMb. The latter suffered more substitutions and were accordingly much more affected. As expected, the patterns of cross-reactivities varied from one monoclonal antibody to another, probably due to some differences in the complementarity residues making up the antibody-combining sites.

Table IV. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Pacific Common Dolphin Mb, Finback Whale Mb, and California Sea Lion Mb and their closest distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb.

A Residue no.	Residues in SpMb	Replaced by			Dist. of A to (closest residue of) site 3 in SpMb[a]
		In PdMb	in FbMb	In CsMb	
1	V	G	—	G	26.00 (Ala-94)
3	S	—	T	—	30.72 (Ala-94)
4	E	D	D	D	30.02 (Ala-94)
5	G	—	A	—	30.95 (Ile-99)
8	Q	—	H	—	34.86 (His-97)
12	H	N	N	N	32.17 (Ile-99)
13	V	—	I	I	26.49 (Ile-99)
15	A	G	—	G	27.29 (His-97)
21	V	L	—	L	20.42 (His-97)
22	A	—	—	V	20.17 (His-97)
27	D	—	—	E	21.96 (Ile-99)
28	I	V	—	V	16.55 (Ile-99)
31	R	—	S	—	19.16 (Pro-100)
35	S	G	G	G	16.48 (Pro-100)
45	R	K	K	K	10.91 (His-97)
51	T	—	—	S	22.84 (Ile-99)
53	A	—	—	D	24.72 (Ile-99)
54	E	D	—	—	20.72 (Ile-99)
57	A	—	—	R	23.32 (Ile-99)
66	V	N	N	K	16.85 (His-97)
74	A	—	G	G	16.03 (His-97)
83	E	D	—	—	18.08 (Ala-94)
109	E	—	D	—	13.25 (Ile-99)
116	H	—	—	Q	27.93 (Ile-99)
118	R	—	—	K	24.88 (Ile-99)
121	G	A	A	—	33.76 (Ile-99)
122	D	E	—	—	32.39 (Ile-99)
127	A	—	—	T	27.92 (Ile-99)
128	Q	—	—	H	24.13 (Pro-100)
129	G	—	A	A	24.07 (Pro-100)
132	N	—	—	K	19.97 (Pro-100)
140	K	—	—	N	17.58 (Ala-94)
147	K	—	—	R	14.67 (Ala-94)
151	Y	F	F	F	**4.24 (Thr-95)**
152	Q	H	—	—	11.49 (Ala-94)

[a] These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of the SpMb structure of Takano (1984).

The studies reported here and in the following papers will afford the opportunity to check whether Mb has discontinuous antigenic sites. These had been surmised to exist in Mb (East *et al.*, 1980, 1982; Berzofsky *et al.*, 1982; Benjamin *et al.*, 1984; Berzofsky and Berkower, 1989) entirely from interpretation of cross-reaction results based on the assumption that every replacement that causes a change in immunochemical reactivity is within the boundaries of the antigenic site. Amino acid residues proposed to constitute discontinuous antigenic sites and which are relevant to antigenic site 3 will be discussed here. Those residues that are relevant to the other sites are discussed in the following papers. Amino acid residues Leu-9, Val-13, Ala-53, His-116, Asp-122, and Gly-124 were proposed to be part of a discontinuous antigenic site (East *et al.*, 1980). The present work shows that the effect of these substitutions is exerted indirectly on the binding of site 3 (as well as other sites, see the following papers). From cross-reactions with two mAbs, Ala-53, His-113 and Ala-74, Ile-142 were interpreted to be in discontinuous antigenic sites (East *et al.*, 1982). The present work has revealed that these substitutions exert their effects rather indirectly on site

Table V. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Dog Mb and Chicken Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) in Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In DgMb	In ChMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
5	G	—	Q	30.95 (Ile-99)
9	L	I	Q	30.23 (Ile-99)
12	H	N	T	32.55 (His-97)
13	V	I	I	26.49 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	T	—	27.31 (His-97)
21	V	L	I	20.43 (His-97)
26	Q	—	H	19.07 (Ile-99)
27	D	E	E	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
30	I	—	M	18.59 (Ile-99)
34	K	—	H	21.08 (Ile-99)
35	S	N	D	16.48 (Pro-100)
41	E	D	D	13.60 (Pro-100)
42	**K**	**—**	**R**	**4.43 (Lys-98)**
45	R	K	K	10.91 (His-97)
48	H	—	G	18.13 (His-97)
53	A	D	P	26.87 (His-97)
54	E	—	D	20.72 (Ile-99)
57	A	G	G	23.32 (Ile-99)
66	V	N	Q	16.85 (His-97)
74	A	G	—	16.03 (His-97)
75	I	—	Q	13.30 (His-97)
85	E	—	D	16.60 (Ala-94)
92	**S**	**—**	**T**	**4.22 (His-97)**
101	I	V	V	8.68 (Pro-100)
109	E	D	—	16.22 (Pro-100)
110	A	—	V	14.19 (Ile-99)
113	H	Q	K	22.04 (Ile-99)
116	H	Q	A	27.93 (Ile-99)
117	S	—	E	24.28 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	S	A	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
124	G	H	—	28.74 (Ile-99)
127	A	T	S	27.92 (Ile-99)
128	Q	E	—	24.13 (Pro-100)
129	G	A	A	24.07 (Pro-100)
132	N	L	K	19.97 (Pro-100)
140	K	N	D	20.50 (Thr-95)
142	I	—	M	8.98 (Ala-94)
144	A	—	S	11.39 (Ala-94)
149	**L**	**—**	**F**	**5.01 (Thr-95)**
151	**Y**	**F**	**F**	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residues in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

Table VI. Amino Acid Residues of Sperm Whale Mb That Undergo Substitution in Badger Mb and Echidna Mb and Their Closest Distances (Å) from Site 3 (Residues 94–100) In Sperm Whale Mb

A Residue no.	Residues in SpMb	Replaced by		Dist. of A to (Closest residue of) site 3 in SpMb[a]
		In BgMb	In EdMb	
1	V	G	G	26.0 (Ala-94)
4	E	D	D	30.02 (Ala-94)
12	H	N	K	32.17 (Ile-99)
15	A	G	G	27.29 (His-97)
19	A	—	T	27.31 (His-97)
21	V	L	I	20.43 (His-97)
22	A	—	T	20.17 (His-97)
27	D	E	—	21.96 (Ile-99)
28	I	V	V	16.55 (Ile-99)
35	S	G	T	16.48 (Pro-100)
45	R	K	K	10.91 (His-97)
51	T	S	—	22.84 (Ile-99)
53	A	D	—	24.72 (Ile-99)
57	A	G	—	23.32 (Ile-99)
59	E	—	A	21.65 (His-97)
66	V	N	G	16.85 (His-97)
67	T	—	V	9.06 (His-97)
74	A	G	S	16.03 (His-97)
81	H	—	Q	24.07 (Ala-94)
82	H	Q	—	15.63 (Ala-94)
100	P	—	S	5.04 (Ile-99)
101	I	V	—	8.68 (Pro-100)
103	Y	—	F	4.92 (Pro-100)
109	E	D	—	16.22 (Pro-100)
112	I	A	—	19.06 (Pro-100)
113	H	Q	—	22.04 (Ile-99)
116	H	Q	Q	27.93 (Ile-99)
118	R	K	K	24.88 (Ile-99)
120	P	—	S	29.87 (Ile-99)
121	G	—	A	33.76 (Ile-99)
122	D	N	—	32.39 (Ile-99)
124	G	A	—	28.82 (Ile-99)
126	D	E	—	31.41 (Ile-99)
129	G	—	A	24.07 (Pro-100)
132	N	K	G	19.97 (Pro-100)
140	K	N	N	17.58 (Ala-94)
142	I	—	I	8.98 (Ala-94)
149	L	—	F	**5.01 (Thr-95)**
151	Y	F	F	**4.24 (Thr-95)**

[a] These values represent the shortest distance between the residue in column A to the site 94–100. Values in bold type indicate distance is less than 7.0 Å. Distances were calculated from the 2.0 Å coordinates of SpMb structure of Takano (1984).

(as well as on other sites as seen in the following papers). Similarly, Glu-4, Glu-83, Ala-144, and Lys-140, which had been proposed to reside within discontinuous sites (Berzofsky *et al.*, 1982), their effects were found here to be in nature indirectly exerted on the reactivity of site 3 (as well as other sites as shown in the following papers). The mAbs employed in those studies (East *et al.*, 1982; Berzofsky *et al.*, 1982) were generated by using whole Mb as the immunogen and, therefore, their site specificity was not known but was totally surmised from cross-reaction studies with Mb variants, each of which had many substitutions, such as the Mbs used in the present work. The results reported here and in the following papers with peptide-elicited mAbs clearly show that such interpretations are not correct.

In conclusion, the results with antisite 3 mAbs have shown that amino acid substitutions outside this

region, which by design were not part of the recognition site of antisite 3 mAbs can exert destructive effects, on the binding of that region. Clearly, therefore, it is incorrect to assume that every mutation affecting the binding of a protein to mAbs should be part of an antigenic site.

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